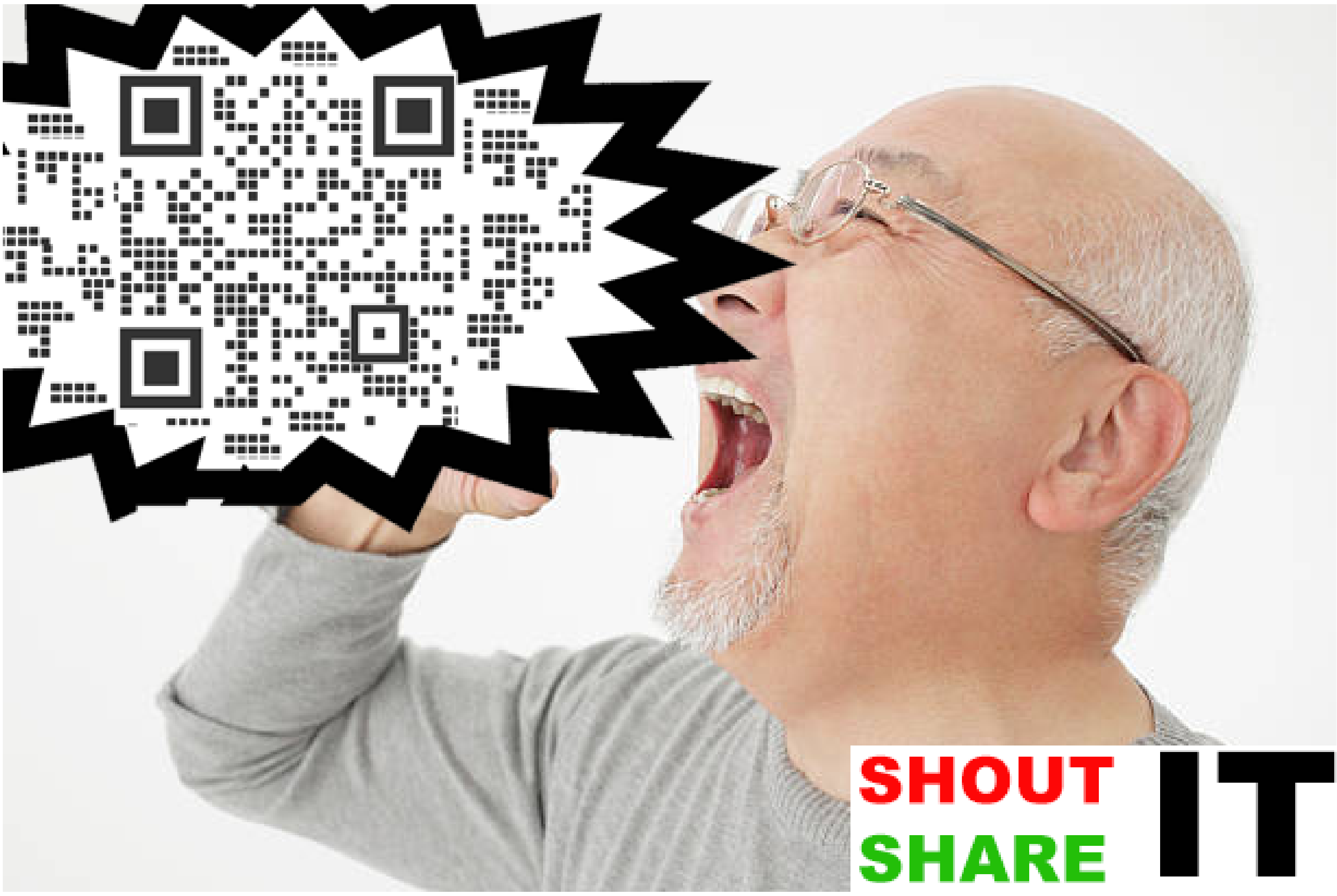
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The Editorial Board requests that manuscripts conform to the requirements set below; those manuscripts which do not conform will be returned to authors for correction before review by the Board.

1. ***Manuscripts.*** Manuscripts must be typed in double spacing (*including* figure legends, foot-notes, bibliography, etc.) on one side of 16- or 20-lb. bond paper, 8½ by 11 inches. They should be carefully proof-read before being submitted and all typographical errors corrected legibly in black ink. Pages should be numbered. A left-hand margin of at least 1½ inches should be allowed.

2. ***Tables, Foot-Notes, Figure Legends, etc.*** Tables should be typed on separate sheets and placed in correct sequence in the text. Because of the high cost of setting such material in type, authors are earnestly requested to limit tabular material as much as possible. Similarly, foot-notes to tables should be avoided wherever possible. If they are essential, they should be indicated by asterisks, daggers, etc., rather than by numbers. Foot-notes are not normally permitted in the body of the text. Such material should be incorporated into the text where appropriate. Explanations of figures should be typed double-spaced and placed on separate sheets at the end of the paper.

3. ***A condensed title*** or running head of no more than 35 letters and spaces should be included.

Continued on Cover Three

Vol. 150, No. 1 February, 1976

THE

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RELATIONSHIP BETWEEN AMMONIA EXCRETION RATES AND HEMOLYMPH NITROGENOUS COMPOUNDS OF A EURYHALINE BIVALVE DURING LOW SALINITY ACCLIMATION [1, 2]

Reference *Biol. Bull.,* **150**: 1–14 (February, 1976)

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Department of Zoology, University of Maryland, College Park, Maryland 20742

Marine invertebrates utilize intracellular free amino acids as the solute for cell volume regulation during environmental salinity stress (marine ciliates, Kaneshiro, Holz and Dunham, 1969; cnidarians, Pierce and Minasian, 1974; sipunculids, Virkar, 1966; polychaetes, Clark, 1968; echinoderms, Lange, 1964; molluscs, Lange, 1963; Pierce, 1971b; Lynch and Wood, 1966; Virkar and Webb, 1970; crustaceans, Vincent-Marique and Gilles, 1970; Gerard and Gilles, 1972). In most species taurine, alanine, glycine, proline, aspartic acid and glutamic acid in some combination, comprise the portion of the amino acid pool utilized for volume regulation (Awapara, 1962).

Although the occurrence of this regulatory process is established, explanations of the biochemical mechanism remain in conflict. The most widely published theory is the mechanism proposed by Florkin and Schoffeniels (1965, 1969), based on data from a few species of euryhaline crustaceans. In these species the activity of the ammonia fixing enzyme glutamic acid dehydrogenase (GDH) is modified by external ion concentrations. An increased environmental salinity causes an increased glutamate synthesis and an accompanying decrease in the rate of ammonia excretion. Glutamate then serves as the starting point for several transamination reactions and the amino acid pool size is increased. A decrease in external salinity produces the reverse effect; amino acid pool size is decreased by catabolism to keto-acids and ammonia. Attempts by Florkin, Schoffeniels and various co-workers to apply this mechanism to all phyla are, on ample evidence, unwarranted. For example, amino acids are released unchanged from the cells of isolated bivalve ventricles during

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hypoosmotic volume regulation instead of being degraded to keto-acids and ammonia (Pierce and Greenberg, 1972). Moreover, while little information is available concerning GDH activity in molluscan species, in the few studies conducted, GDH either was not found or had very low activities. Possibly the pathways of amino acid metabolism in molluscs are unique, or perhaps the properties of molluscan GDH are unusual, making detection difficult under the assay conditions used to date (Campbell and Bishop, 1970). However, in view of existing data, cell volume regulation in molluscs during low salinity stress is accomplished by release of intracellular amino acids rather than by a catabolic process. Nonetheless, the whole animal excretion patterns of bivalves (Lum and Hammen, 1964; Emerson, 1969) as well as crustaceans (Jeuniaux and Florkin, 1961; Haberfield, 1971) are ammonotelic.

The combined results of these molluscan and crustacean studies have led us to the hypothesis that during low salinity acclimation by intact bivalves, cell volume is maintained by an efflux of intracellular amino acids into the body fluids. Only after the amino acids have left the intracellular osmotic solute pool does deamination occur, resulting in an increased ammonia excretion rate.

Only one study of changes in nitrogen excretion rate of intact bivalves during salinity stress has been reported (Emerson, 1969) and the results were inconclusive. *Macoma inconspicua* increased ammonia excretion upon transfer from 100% to 50% sea water, while the rate of ammonia loss from *Mya arenaria* remained unchanged. Moreover, no measurements of blood ammonia or amino acid concentration have been correlated with ammonia excretion rates of bivalves acclimating to different salinities. Thus, the whole animal nitrogen excretion response during salinity stress has not yet been established in molluscs.

We have investigated the effect of lowered external salinity on whole animal ammonia excretion rates and on concentrations of some hemolymph nitrogenous compounds of a euryhaline bivalve, *Modiolus demissus demissus* Dillwyn, both during and following acclimation. The results show that following a decrease in external salinity, an increase in hemolymph amino acid concentrations occurs which coincides with a decrease in the tissue free amino acid pool. As acclimation proceeds, blood amino acid concentrations level off in company with an increase in both blood ammonia concentration and external ammonia release. Finally, hemolymph amino acid concentrations return toward control levels, followed by a decrease in hemolymph ammonia concentration and external ammonia excretion rate.

MATERIALS AND METHODS

Animals

Modiolus demissus were collected from a salt marsh on the Chincoteague Bay side of Assateague Island, Maryland. They were kept in aquaria containing aerated artificial sea water (Instant Ocean; salinity = 35‰ = 1020 mOsm/kg H_2O = full strength sea water, hereafter) in the laboratory (22° ± 1° C) at College Park, Maryland. All animals were acclimated to full strength sea water for at least three weeks before being used experimentally.

Preparation of animals

Twenty-four hours before the start of each experiment, animals were removed from the main aquarium and the valves were scrubbed thoroughly with a brush to remove fouling organisms. The mussels were then propped open with pieces of glass tubing inserted between the ventral valve margins. This procedure prevented the animals from closing and thus ensured constant exposure of the soft parts of the animals to the experimental sea water conditions. The propped-open animals were kept together in a container of aerated Millipore filtered (0.45 μ) full strength sea water for 24 hours.

Ammonia excretion rates during acclimation

Following the preparation described above, propped-open *M. demissus* were placed individually into beakers containing 700 ml of either filtered full strength sea water (control) or filtered 50% sea water (510 mOsm/kg H_2O) (experimental) at 22° ± 1° C. Twelve animals were tested in each salinity. After 12 hours, each animal was removed from the test solution, the contents of the mantle cavity drained into the test solution and the animal placed into 700 ml of fresh sea water of the same osmotic concentration. Using this transfer technique, animals were placed in fresh sea water every 12 hours for 84 hours. Sea water samples were removed from the beakers and assayed for ammonia nitrogen at the 12, 24, 36, 60 and 84 hour transfer times, following the phenolhypochlorite method as modified by Solorzano (1969). In this procedure, ammonia is reacted with phenol and hypochlorite at a high pH to produce indophenol, a blue-colored product. The indophenol concentration was determined spectrophotometrically (Spectronic 20; Bausch and Lomb) at 640 nm and converted to ammonia concentration by comparison with a standard curve. At the end of the experiment, the soft parts of each mussel were removed from the shell, freeze-dried and weighed to obtain a dry tissue weight.

Since preliminary experiments, as well as those of other investigators (Hammen, 1968; Emerson, 1969), indicated an influence of body weight on excretion rates within a treatment group, this effect was removed as follows: a pooled within-treatment regression line was calculated using data from the 84-hour treatment groups; the slope of this line was then used to adjust individual excretion rate values at all time periods to the grand mean animal weight. The following equation was used: $Y_{\text{adjusted}} = Y_{\text{measured}} - b$ (individual weight − grand mean weight), where Y is the ammonia excretion rate and b is the slope of the pooled within-treatment regression line. Adjusted means of treatment groups at each time period were then calculated.

For this experiment, and for all subsequent experiments unless otherwise specified, means of treatment groups at each time period were compared statistically using the Student's t-test. P-values less than 5% were accepted.

Ammonia production of isolated valves

An additional control experiment was done to determine the amount of ammonia production which could be attributed to nonmussel sources (*e.g.*, bacteria) remaining on the valves of the scrubbed mussels. Animals acclimated to full strength sea water were dissected from their scrubbed shells. The valves (five pairs per treatment group) were then treated as experimental and control animals in an experiment identical to the 84-hour time course study described above.

Ammonia concentration in hemolymph during acclimation

The concentration of ammonia in the blood of propped-open experimental and control mussels was measured 0, 4, 12, 24 and 60 hours following initial exposure of animals to test sea water in an experiment conducted as previously described. However, since animals had to be sacrificed to obtain blood samples, a different group of mussels (twelve per treatment group) was used to obtain blood ammonia values for each time period.

Each blood sample was obtained by removing one valve of a mussel, blotting the mantle and visceral mass dry with filter paper and slashing the mantle and adductor muscles with a scalpel blade. The anterior end of the animal was then placed into the mouth of a 50 ml centrifuge tube and both the animal and tube wrapped tightly with Parafilm to prevent evaporation. Hemolymph was allowed to drain from the cut tissue surfaces into the tube for 20–35 minutes at 4° C. The collected blood was then centrifuged at 3000 *g* for ten minutes to remove any contaminating tissue debris. Hemolymph collected by this technique is osmotically equivalent to hemolymph removed directly from the circulatory system by syringe (Pierce, 1970). Therefore, significant contamination of the blood from intracellular sources is unlikely. Blood samples were appropriately diluted with deionized water to a final volume of 5 ml and then analyzed for ammonia nitrogen as described above.

Free amino acid concentrations in hemolymph during acclimation

The concentrations of amino acids in the blood of propped-open experimental and control mussels were measured 0, 2, 4, 12, 24 and 60 hours following introduction of animals into test sea water. Test salinities and water change intervals were the same as in the previously described experiments. At the end of each time period blood samples were collected from six mussels in each salinity. Appropriate volumes of blood were diluted with glass distilled water to a final volume of 1 ml. Amino acids were extracted and proteins precipitated by adding 1 ml of 80% ethanol to each diluted blood sample. This mixture was brought to a boil in a water bath, allowed to extract below the boiling point for 30 minutes and then centrifuged at 20,000 *g* for 30 minutes. The supernatant solution was saved and the precipitate washed once with 2 ml of 40% ethanol and centrifuged again. The supernatant solutions from both centrifugations were pooled and lyophilized. The resulting residue was dissolved in an appropriate volume of sodium citrate buffer (pH 2.2) and analyzed for acidic and neutral amino acids with an amino acid analyzer (JEOL—Model JLC-6AH). Previous investigators have shown that acidic and neutral amino acids are the major constituents of the free amino acid pools of *Modiolus* blood and tissues and are also the only intracellular amino acids utilized for cell volume regulation during salinity stress (Pierce 1971b; Pierce and Greenberg, 1972, 1973).

The amino acid analysis of blood samples consistently revealed a peak with the same elution time as that of the urea contained in our amino acid standard mixture. Since urea excretion is uncommon among bivalves (Campbell and Bishop, 1970), an experiment was performed to determine whether or not this peak was actually urea. Urease (Sigma Chemical Co.) was dissolved in phosphate buffer prepared according to the method of Welsh, Smith, and Kammer (1968). A blood sample was divided into four aliquots. A known amount of urea was added to one aliquot, urea plus urease was added to the second, urease alone was added to the third and the fourth aliquot was left unaltered. All four solutions were incubated for 30 minutes at 30° C. Ethanol extraction and amino acid analysis were then completed as described above.

Free amino acid concentrations in tissues during acclimation

The concentrations of amino acids in specific tissues were measured at various times following introduction of propped-open animals into test sea water. Ventricles were removed at the end of 4, 12 and 24 hour exposures. Mantle samples were taken at 4, 12, 24 and 60 hours. At each time period tissues were removed from 32 animals (16 experimental and 16 control), frozen on dry ice and lyophilized. The dry tissue pieces, in groups of four, were weighed and then homogenized and extracted in 80% ethanol as previously described. Concentrations of acidic and neutral amino acids were determined with the amino acid analyzer.

Natural acclimation of mussels to low salinity

A large group of mussels was placed into 50% sea water and allowed to acclimate to that salinity for at least five weeks without being propped open. This period of time is more than adequate to ensure complete osmotic acclimation of *M. demissus* to any non-lethal salinity (Pierce, 1970).

Ammonia excretion rates following acclimation

At the end of the acclimation period, 22 animals were scrubbed, propped open and placed into Millipore filtered 50% sea water for 24 hours. The mussels were then transferred into individual beakers of fresh 50% sea water. This water was changed after 12 hours and the concentration of ammonia collected during the 12 to 24 hour interval was determined. Similar measurements in full strength sea water were made on 20 animals acclimated to full strength sea water for five weeks. Analysis of covariance was employed to remove the effect of weight on excretion rates and to compare the means of the experimental and control groups.

Free amino acid concentrations in hemolymph following acclimation

At the end of the five week acclimation period, blood samples were removed from twelve mussels and analyzed for amino acids according to the methods presented above. Blood amino acid concentrations were also determined for twelve animals naturally acclimated to full strength sea water for five weeks.

RESULTS

Ammonia excretion rates during and following acclimation

In all the experiments described below, a significant correlation ($P < 0.01$) was found between ammonia excretion rate and dry tissue weight. Larger animals had smaller excretion rates irrespective of salinity (Fig. 1). To compensate for this

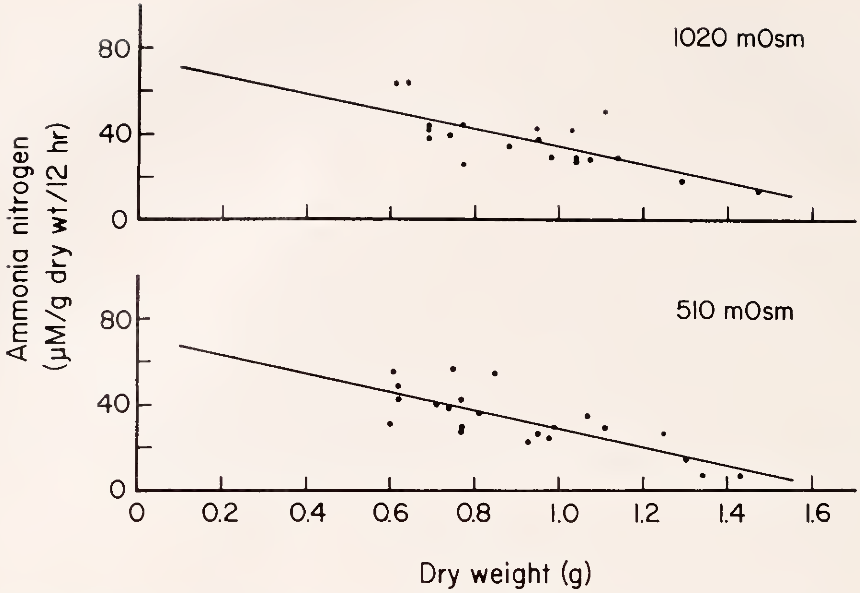

FIGURE 1. Linear regression analyses of ammonia excretion rate as a function of dry tissue weight for propped-open *M. demissus* in full strength sea water (1020 mOsm/kg H_2O) and 50% sea water (510 mOsm/kg H_2O). Each point represents one animal. Mussels were previously acclimated to their respective salinities for five weeks. Regression line formulae are: full strength sea water, $Y = -40.88x + 74.64$, $r = -0.719$; 50% sea water, $Y = -42.70x + 71.23$, $r = -0.777$.

weight effect, all measured ammonia excretion rates were statistically adjusted (see Methods) to the grand mean dry weight of the mussels used in each experiment. The data presented below are the adjusted values.

During low salinity acclimation, no significant difference was found between excretion rates of control and experimental animals during the first 12 hours of exposure to the test salinity (Fig. 2). However, during the 12 to 24 hour, 24 to 36 hour and 48 to 60 hour time periods, a significantly higher ammonia excretion rate occurred in the experimental animals ($P < 0.01$). The excretion rate of animals in low salinity reached a maximum of 51.7 µM NH_3-N/g dry wt/12 hours during the 12 to 24 hour collection period, leveled off during the 24 to 36 hour interval and then gradually began to decline toward control rates. However, the mean excretion rate of the experimental animals was still greater than that of the control animals during the 72 to 84 hour time period ($P < 0.05$).

The mean ammonia excretion rates (± standard errors) of mussels naturally acclimated for five weeks to full strength and 50% sea water were 37.2 ± 2.01 and 32.1 ± 1.92 µM NH_3-N/g dry wt/12 hours, respectively. The difference between the two excretion rates was not significant.

Concentrations of ammonia produced by isolated valves of *M. demissus* during the 84 hour time course of low salinity exposure are listed in Table I. The valves in 50% sea water produced more ammonia than those in full strength sea water at

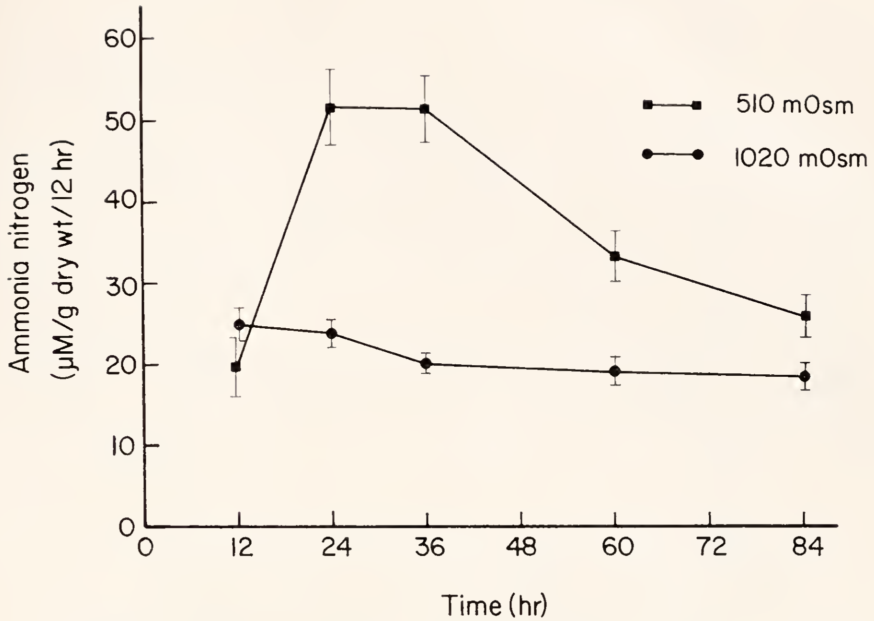

FIGURE 2. Time course of changes in ammonia excretion rates of propped-open *M. demissus* during low salinity acclimation. Mussels were previously acclimated to full strength sea water. Each point represents the mean excretion rate of twelve animals during the previous 12 hour interval. Error bars represent standard error.

all time intervals except the first one (0–12 hours). However, the quantities of ammonia produced by the isolated valves were in all cases less than the standard errors of whole animal excretion rates measured at the corresponding time intervals. Therefore, these nonmussel sources of ammonia were not a source of contamination.

Ammonia concentration in hemolymph during acclimation

Blood ammonia concentrations in control and experimental animals were not significantly different following both 4 and 12 hours exposure to test sea water

TABLE I

Ammonia production of isolated valves of M. demissus. *Each value represents the mean ammonia production of five pairs of valves during the previous 12-hour interval.*

Time (hour)	Ammonia production	
	1020 mOsm/kg H_2O	510 mOsm/kg H_2O
12	1.7 (±0.07)*	1.3 (±0.43)
24	1.2 (±0.07)	1.6 (±0.21)
36	1.1 (±0.07)	1.3 (±0.21)
60	0.9 (±0.07)	2.7 (±0.29)
84	1.0 (±0.07)	1.9 (±0.36)

* µM NH_3-N/ 12 hour (±SE).

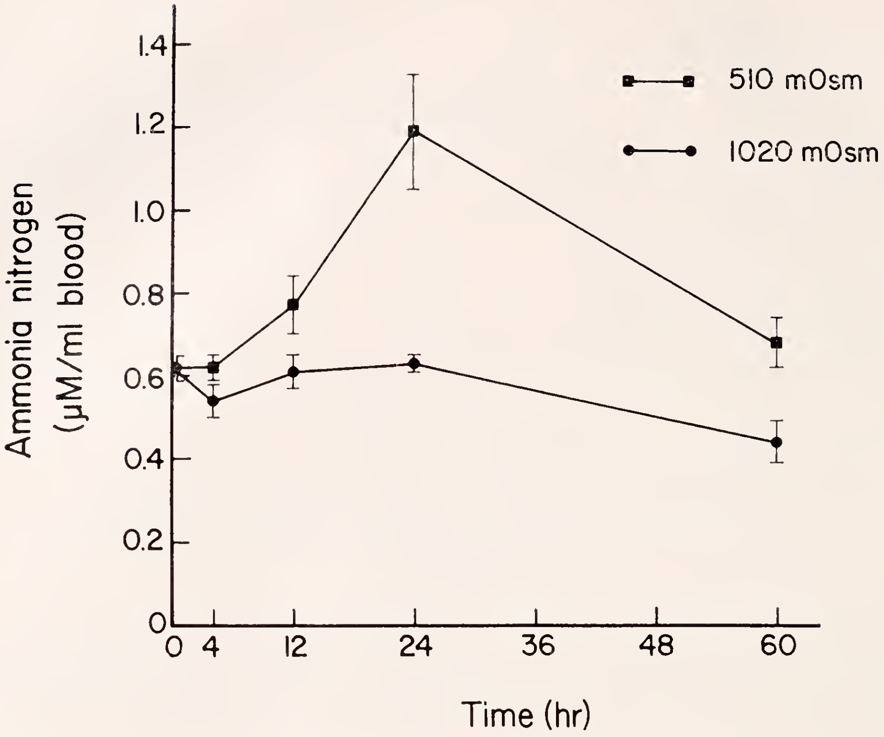

FIGURE 3. Time course of changes in hemolymph ammonia concentrations of propped-open *M. demissus* during low salinity acclimation. Mussels were previously acclimated to full strength sea water. Points are mean values from twelve animals. Error bars designate standard error.

(Fig. 3). After 24 hours, however, experimental animals had a significantly higher concentration of ammonia in the blood than did the control animals ($P < 0.01$), reaching a maximum concentration of 1.2 μM NH_3-N/ml blood. Analysis of 60-hour samples showed that ammonia concentrations in the experimental animals were returning toward control levels by this time but were still significantly higher ($P < 0.01$).

Free amino acid concentrations in hemolymph during and following acclimation

Urea was not found in the hemolymph of *M. demissus.* Amino acid analyses of unaltered blood, a mixture of blood and urease and a mixture of blood, urea and urease resulted in identical chromatograms of the compound eluting at the same time as urea in our standard amino acid mixture. Therefore, this compound is not urea and remains unidentified.

During acclimation the changes in total free amino acid concentration in the blood of experimental mussels (Fig. 4) followed a pattern similar to the changes in blood ammonia, except that the elevation of amino acid concentration preceded the rise in blood ammonia (Fig. 3). After both 4 and 12 hours exposure to test

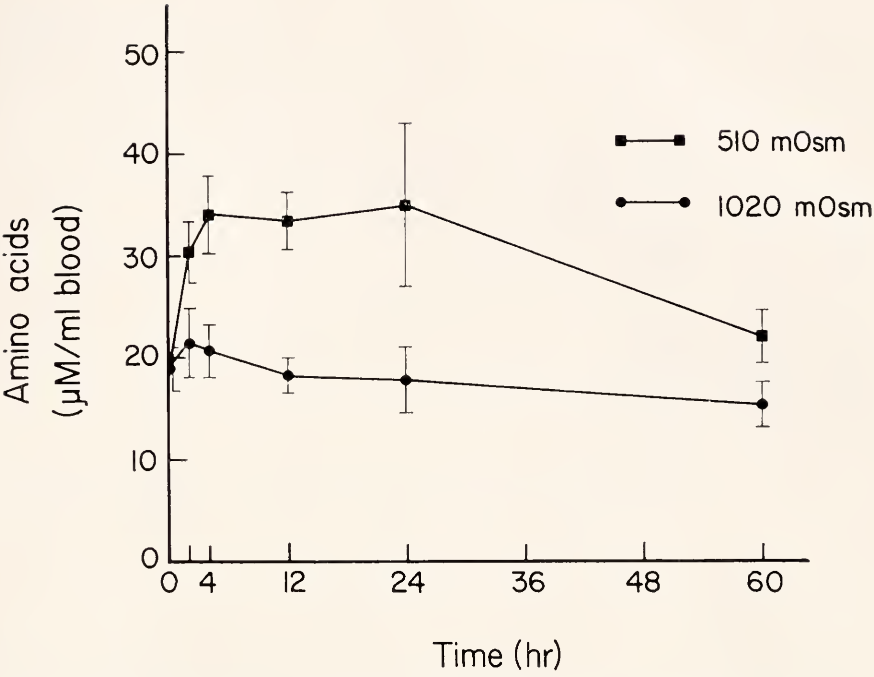

FIGURE 4. Time course of changes in total free amino acid concentrations in the hemolymph of propped-open *M. demissus* during low salinity acclimation. Mussels were previously acclimated to full strength sea water. Points are mean values from six animals. Error bars represent standard error.

salinities, the total free amino acid concentration of the blood was significantly higher in the experimental mussels (4 hours, $P < 0.05$; 12 hours, $P < 0.01$). At the 24 hour sampling period the mean free amino acid concentration in the blood of experimental animals was still much higher than the control mean concentration. However, the variation in concentrations within the experimental group was large enough so that no statistical difference could be found between the two groups of mussels. After 60 hours the total free amino acid concentration in blood of the animals in low salinity had declined toward control values and was not significantly different.

Following complete acclimation to low salinity, mussels have total blood amino acid concentrations much lower than those of animals acclimated to full strength sea water. The mean total free amino acid concentrations (± standard errors) in blood of animals acclimated for five weeks to full strength and 50% sea water were 18.9 ± 2.15 and 10.6 ± 1.08 μM amino acids/ml blood, respectively.

Free amino acid concentrations in tissues during acclimation

The total free amino acid pool in ventricles of mussels acclimating to 50% sea water decreased steadily with time (Fig. 5). Mean values of total amino acids

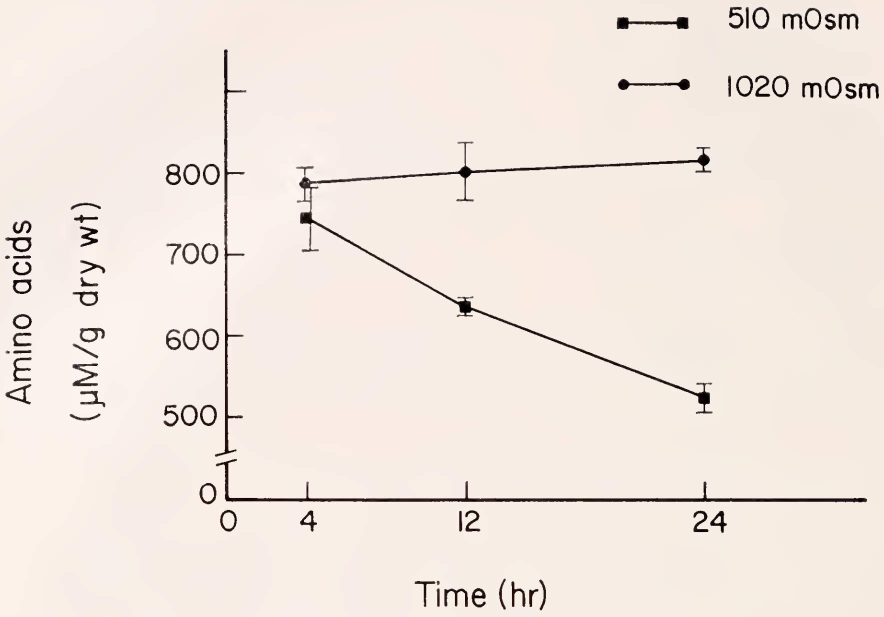

FIGURE 5. Time course of changes in total intracellular free amino acid concentrations in ventricles removed from *M. demissus* during low salinity acclimation. Mussels were previously acclimated to full strength sea water. Each point represents the mean of four tissue homogenates, each containing four ventricles. Error bars designate standard error.

in the ventricles of experimental animals were lower than in ventricles of control animals at all times investigated. These differences were significant ($P < 0.01$) at the 12 and 24 hour sampling periods.

Concentrations of total free amino acids in mantle tissue of experimental and control animals at various times during low salinity acclimation are presented in Table II. There was no significant difference between the control and experimental amino acid pools at any time period examined.

TABLE II

Changes in total intracellular free amino acid pool in mantle tissue of propped-open M. demissus *during low salinity acclimation. Each value represents the mean of four tissue homogenates each containing mantle tissue from four mussels.*

Time (hour)	Amino acid concentration	
	1020 mOsm/kg H_2O	510 mOsm/kg H_2O
4	675.4 (±18.50)*	712.6 (±18.23)
12	662.3 (±33.88)	684.9 (±31.77)
24	684.4 (±34.68)	706.9 (±29.27)
60	718.3 (±45.76)	705.6 (±30.51)

* μM/g dry tissue wt. (±SE).

DISCUSSION

During volume regulation of isolated ventricles of *M. demissus* to low salinity, free amino acids rapidly efflux from individual cells along with osmotically obligated water (Pierce and Greenberg, 1972). The data presented here show clearly that this same mechanism operates in the whole animal during low salinity acclimation. Namely, in response to lowered external osmotic pressure, cell swelling is controlled by the rapid release of free amino acids from the intracellular pool into the hemolymph. The time course of the elevation in blood amino acid concentration reported here corresponds closely to the rate of change in hemolymph osmolality in propped-open *Mytilus edulis,* a closely related species, over a similar salinity range (Costa, 1976). The amino acids released into the hemolymph are not excreted from the whole animal. Instead, several hours after the rise in hemolymph amino acid concentration, blood ammonia levels increase coincident with increased external ammonia excretion rates. As acclimation proceeds, the amino acid concentration of the blood decreases back toward control levels, followed by a decrease in blood ammonia. Finally, external ammonia excretion rates return to control levels. Therefore, it appears that the amino acids are being degraded, rather than excreted, some time after leaving the cell as osmotic solute.

The exact time relationship between increased blood ammonia concentrations and elevated external ammonia excretion rates is not clear. Blood ammonia concentrations first appear elevated at 24 hours following the salinity change, which is both considerably after the initial amino acid release and in the midst of the peak of external ammonia excretion rates (12–36 hours). We did not measure either blood ammonia or external ammonia at intermediate time intervals between 12 and 24 hours. However, since excess ammonia usually diffuses rapidly out of the tissues of aquatic animals these two phenomena may coincide.

The ammonia excretion rates of mussels acclimated to low salinity are the same as those of animals acclimated to full strength sea water. Moreover, the total free amino acid pool of the blood of the low salinity mussels is lower than that of animals in full strength sea water. Therefore, the high levels of hemolymph amino acids and the elevated ammonia excretion rates observed during acclimation are transitory and do not persist after acclimation to low salinity is complete.

In spite of the transient increase in ammonia excretion rates by *M. demissus* during low salinity acclimation, the mechanism of intracellular free amino acid regulation is unlike that which has been proposed for Crustacea (Florkin and Schoffeniels, 1965, 1969; see introduction). First, it is obvious that blood free amino acid levels in acclimating mussels become elevated before blood ammonia concentrations increase. Therefore, in the whole animal amino acids are released intact from cells into the blood, as Pierce and Greenberg (1972) have demonstrated with isolated tissue, rather than being degraded intracellularly. In fact, similar changes in blood amino acid concentrations have also been observed in crustaceans (Vincent-Marique and Gilles, 1970; Haberfield, 1971; Gerard and Gilles, 1972), as well as in several polychaete annelids (Clark, 1968) and *Mytilus edulis*, another mussel (Potts, 1958; Bricteux-Grégoire, Duchâteau-Bosson, Jeuniaux, and Florkin, 1964). Second, since peak concentrations of ammonia both in the blood and outside the animal follow the elevation of free amino acid concentrations in the blood, the increased production is most likely due to degradation of amino acids by a particular organ of the body following the volume regulation response. A similar mechanism has been suggested to reconcile proline release from cells of the crab *Eriocheir sinensis* and the lack of external proline excretion during low salinity stress (Vincent-Marique and Gilles, 1970).

The free amino acid pool of the mantle tissue is reduced in *M. demissus* completely acclimated to lower salinity (Pierce, 1971b). However, while the free amino acid concentration of the ventricle is already decreased 12 hours after the animal is exposed to low salinity, the pool in the mantle has not begun to decrease, even after 60 hours. The mantle may simply have a much lower permeability to water and ions than other tissues. On the other hand, the mantle may well be a site of accumulation and degradation of amino acids released by other tissues during salinity stress. Read (1962) and Awapara and Campbell (1964) have shown the presence of amino-transferase activity in the mantle tissue of various bivalves. Furthermore, the mantle is highly vascularized and, since it presents a large surface area in direct contact with the external environment, a rapid release of large quantities of toxic ammonia could be achieved as soon as the ammonia is produced. Confirmation of this hypothesis awaits considerable experimental evidence.

Finally, the ammonia excretion rates determined in this study are 10 to 15 times greater than those determined for *M. demissus* in full strength sea water by previous investigators (Lum and Hammen, 1964). A number of factors may have contributed to the large discrepancy between excretion rates measured in the two studies. First of all, Lum and Hammen did not prop open the shells of the mussels nor, apparently, did they drain the mantle cavity before water samples were taken for ammonia analysis. Second, Lum and Hammen placed six to ten animals together in 50 ml of water and left them in that same water for 24 hours prior to analyzing the water for ammonia. Needham (1957) has demonstrated depression of nitrogen output when animals are grouped in a limited volume of medium. Moreover, ammonia accumulation in the external environment inhibits further production of ammonia by organisms (Needham, 1957; Lum and Hammen, 1964). Finally, in calculating excretion rates Lum and Hammen did not use measured tissue weights. Rather the tissue weights were calculated from the whole animal weight (including shell), using a correction factor determined in a previous experiment (Hammen, Hanlon, and Lum, 1962). This correction factor assumes that the dry tissue weight equals 35% of the wet weight (tissue and fluid) of the whole animal. This percentage is 2 to 3.7 times higher than measured values determined by other investigators (Martin, Harrison, Huston, and Stewart, 1958; Pierce, 1971a). Thus, the estimated weights used by Lum and Hammen (1964) may have been larger than the true animal dry weight and therefore the ammonia excretion rates calculated would be smaller than the actual excretion rate.

SUMMARY

1. Free amino acid concentrations in the hemolymph increase rapidly in company with a decrease in the tissue free amino acid pool of propped-open *M. demissus* exposed to low salinity.

2. As acclimation proceeds, blood amino acid concentrations level off and an increase in both hemolymph ammonia concentration and external ammonia excretion occurs.

3. Finally, hemolymph amino acid concentrations decrease toward control levels, followed by hemolymph ammonia concentration and external ammonia excretion rates.

4. Following complete acclimation of mussels to high or low salinity, ammonia excretion rates are the same. However, hemolymph free amino acid concentrations are lower in animals adapted to low salinity.

5. These results show that during acclimation of mussels to low salinity, free amino acids are released intact from the cells into the hemolymph. The subsequent transitory increase in hemolymph ammonia concentrations and external ammonia excretion rates is probably due to degradation of the effluxed amino acids by some specific organ of the body.

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CIRCULATORY ADAPTATIONS TO THE OXYGEN MINIMUM LAYER IN THE BATHYPELAGIC MYSID *GNATHOPHAUSIA INGENS*

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Zones of minimum oxygen are found at intermediate depths in most of the world's oceans and although the dissolved oxygen in some of these "oxygen minimum layers" is considerably less than 0.5 ml/l, populations of metazoans exist there (Schmidt, 1925; Sewell and Fage, 1948; Banse, 1964). Previous studies have shown that crustaceans which live in such minimum layers are unusually effective at removing oxygen from water (Teal and Carey, 1967; Childress, 1968, 1969, 1971, 1975). The respiratory physiology of one of these organisms, the lophogastrid mysid, *Gnathophausia ingens* has been studied in some detail by Childress (1968, 1969, 1971). These reports show that *G. ingens* is able to live aerobically at oxygen partial pressures of 6 mm Hg at an oxygen consumption rate of 0.8 mgO_2/Kg wet wt/min. The ability of this species to maintain a very high flow rate of water over its gills (up to 8 ml/g wet wt/min), while removing 50 to 80 per cent of the oxygen is apparently an important factor in this animal's remarkable aerobic ability (Childress, 1971). The major remaining questions concern the removal of O_2 from the respiratory stream, its passage through the gills and its movement to the tissues by way of the circulatory system.

The relationship between respiratory characteristics and the internal circulation has remained largely unstudied in crustacea. Present understanding of crustacean haemodynamics is based primarily upon anatomical studies of several species and a small number of direct measurements, such as the blood pressure measurements made in crabs and lobsters (Dubuisson, 1928; Drach, 1939; Burger and Smythe, 1953; Blatchford, 1971). The use in intact crustaceans of newer, more sensitive techniques for the measurement of internal pressure and fluid velocities (techniques previously utilized primarily in fishes and other vertebrates) has now permitted more detailed analysis of crustacean circulatory dynamics (Belman, 1975; Cobbold, 1974). In this continued examination of the unique respiratory abilities of *G. ingens*, we have used such techniques to study the blood circulatory system of this species.

MATERIALS AND METHODS

Most of the specimens of *Gnathophausia ingens* used were taken in the basins off southern California with a 3.1 meter square Tucker trawl from the R/V OCONOSTOTA. Immediately after they reached the surface, the animals were placed in four liter polyethylene jars containing sea water at 3° C. The animals were

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transported in refrigerated containers to the Marine Laboratory at the University of California, Santa Barbara, and maintained at either 4.5 or 5.5° C. Individual specimens of *G. ingens* have survived in this laboratory for more than two years on a diet of salmon and lobster. All of the experimental animals were sexually immature individuals of undetermined sex and had a wet weight of less than 18 g.

Blood pressure and velocity

Blood pressure determinations were made on animals restrained in a chamber of sea water held at 5° C. Blood pressures were monitored with Statham 23BB pressure transducers connected *via* polyethylene tubing to #27 gauge hypodermic needles inserted into the blood system. Needle placement was facilitated with micromanipulators and the needle positions checked by dissection following the experiment. Pressure output was recorded on either an inkwriting recorder or oscillograph. All values given below represent pressure in cm H_2O above ambient pressure.

Blood flow velocities were measured directly with a Paul Beckman Associates' blood fluid velocity probe. This device consists of a #25 gauge needle with a heated thermocouple at the tip and measures fluid velocity by means of heat dissipation from the heated thermocouple. Blood velocity was measured in the heart, dorsal abdominal artery and hepatopancreas. Using a micromanipulator, the probe was placed into the vessel or tissue of interest after being gently pushed through either tiny holes in the carapace or the articular membrane between somites. The animals were lightly restrained under the same conditions as for the pressure measurements.

The flow-probe was calibrated by direct volumetric readings when placed in a polyethylene tube of known diameter. The calibration fluid was sea water at 5° C. As the density of crustacean blood is only slightly greater than that of sea water at the same temperature (Maynard, 1960a), no correction factor was introduced. The response of the probe is logarithmic. The direct volumetric readings were used in preparing linear velocity calibration charts for each experiment. Determinations were made of blood velocity in cm/sec from the strip-chart recordings of output from the flow-probe using these calibration charts.

Since the time period of the acceleration of the blood during a systolic contraction of the heart is on the order of 300 milliseconds or more, it was necessary to determine as accurately as possible the response-time of the flow probe. This was done by comparing the response of the flow-probe to that of a Statham 23BB pressure transducer. The flow-probe and a #25 gauge hypodermic needle connected *via* polyurethane tubing to the transducer were placed in a 0.15 cm diameter polyethylene tube connected to a peristaltic pump (60 strokes/min). The outputs from each were recorded simultaneously on a Beckman Instruments RS Dynograph at a chart-speed of 125 mm/sec. While there was some variation in response-time of the flow-probe, the lag behind the pressure trace was never more than 15 milliseconds. This indicates that the maxium error due to response-time is about 5% or less and that the flow-probe can follow the flow changes occurring in the heart of this species.

Following each experiment in which blood velocity was measured, the animal was dissected, placement of the flow-probe checked, and the diameters of the heart or other vessel measured at the point where flow was measured.

In both the blood velocity and blood pressure experiments, measurements were made at both high and low oxygen concentrations. These were carried out by sealing the chamber in which the animal was restrained and then slowly introducing N_2-saturated water at the experimental temperature. The O_2 content of the water in the chamber was reduced to levels lower than 1.0 ml/l and held there for several hours. Oxygen partial pressure was continuously monitored with a Clark-type oxygen electrode.

Gill surface area

As previously noted (Childress, 1971), the gills of *G. ingens* are so irregular as to preclude any possibility of measuring their surface area by the usual methods. The following method was devised to deal with this difficulty. Gill surface area was determined by measuring the density of cast exoskeletal material (carapace), the average thickness of the gill exoskeleton from cast exoskeletons and the weight of the gills from cast exoskeletons. From the density (D), thickness (T), and total weight (W) of the cast gills, the gill area was computed by the relationship: area $= W/DT$.

The thickness of the gill exoskeleton was measured using a Filar eyepiece calibrated with a stage-micrometer. The weights were all taken as ash-free dry weight, ash forming about the same fraction of both gill and carapace exoskeleton.

Heart rate (EKG)

Heart rates (EKG's) were measured in response to low oxygen using paired silver electrodes placed on the heart through small holes in the carapace and glued in place with dental cement. The electrical signal was amplified with a Grass P-15 A-C pre-amplifier and recorded on an oscillograph. The animal was lightly restrained in the cooling-jacketed chamber described above and lowered oxygen levels were obtained by flowing N_2-saturated water through the chamber. Oxygen partial pressure was monitored with a Clark-type oxygen electrode.

Heart volume

Heart volume was calculated from the geometry of the heart. Measurements were made of heart diameter at four places along the longitudinal axis and of the heart length from the posterior valve to the anterior exit to the median cephalic artery in five animals. Three kinds of measurements were taken. First the diameters were determined at maximum filling by observing the heart in the intact animal. The dorsal carapace was then removed and while the heart continued to beat, a second set of measurements was made. Finally, the heart was dissected out of the animal, measured, and weighed. All three measurements agreed within 0.1 mm.

RESULTS

Anatomy of the blood vascular system

Present knowledge of crustacean blood systems is not comprehensive. Only a few species are well known, and for most of these, only the arterial system has been examined in any detail (Maynard, 1960a; Pillai, 1965; Seaton, 1971). No detailed account of the anatomy of the blood system in the Mysidacea exists, although Delage (1883) and Alexandrowicz (1955) provide some information on the structure of the heart and arterial system in *Mysis* and *Praunus flexuosus,* respectively.

The anatomy of the arterial system and of the return pathways in *G. ingens* was examined in eight individuals. Injection of Evans Blue into the heart or pericardial chamber caused this dye to be circulated throughout the arterial system and rendered the transparent vessels visible. Similar injections of dye into the ventral sinus channels in living animals enabled the return pathways to be mapped. In three individuals, a silicone rubber injection material (*Microfil,* Canton Biomedical Products) was injected into the arterial system, allowed to set, and the resultant casts dissected free and examined.

The heart. The heart in *G. ingens* is a single chamber, generally tubular in shape, extending throughout the posterior half of the thorax. It is suspended within the pericardial sinus although tending to lie toward the lower surface of the chamber. Ligaments attach both dorsally and laterally along its entire length. Structurally the heart appears similar to that in other mysids (Gadzikiewicz, 1905). Observations of the beating heart *in vivo* suggest that the contraction begins near the ostial openings and proceeds as a peristaltic-like wave in both the anterior and posterior directions. The temporal sequence in the normal heartbeat therefore is similar to that in *Holopedium* (Storch, 1931). Systole and diastole are apparently not discrete events temporally and blood enters the heart through the ostia while systolic contraction proceeds distally.

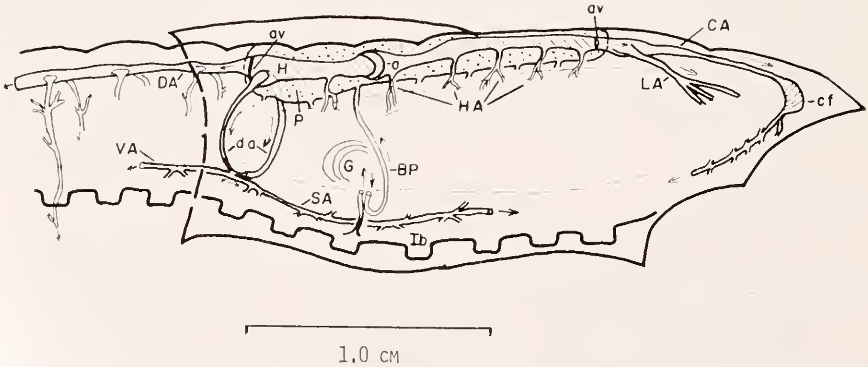

FIGURE 1. Arterial system of *Gnathophausia ingens.* Arrows denote direction of blood flow. H represents heart; av, arterial valves; o, ostial openings; DA, dorsal abdominal artery; da, descending arteries; SA, sternal artery; VA, ventral abdominal artery; HA, hepatic arteries; CA, median cephalic artery; cf, *cor frontale*; LA, lateral anterior arteries; Ib, infrabranchial sinus; BP, branchiopericardial vessels; and P, pericardium.

Arterial system. The arterial system of *G. ingens* is depicted in Figure 1. Blood leaves the heart through four main arteries and a number of smaller ones. Posteriorly, the dorsal abdominal artery (DA), of diameter nearly equal that of the heart itself, exists through a large valve. This artery passes along the dorsal wall of the abdomen with paired branches in each abdominal segment passing ventrally to divide further and supply the muscle of the abdominal wall and the pleopods. The descending arteries (da) exit the heart just anterior to its junction with the dorsal abdominal artery. The descending arteries pass laterally and then continue ventrally to rejoin, penetrate the center of the ventral ganglion, and terminate in the large horizontal, sternal artery (SA). The sternal artery extends anteriorly symmetrically giving rise to branches serving the periopods and more anteriorly, the maxillae. Posteriorly, the sternal artery continues along the ventral nerve cord becoming the ventral abdominal artery (VA).

Along the length of the heart, a series of small arteries arise which bifurcate and extend into the hepatopancreas. These are termed the hepatic arteries (HA). The supply of blood to the hepatopancreas is through multiple branches of these arteries and branches of the sternal artery, posterior abdominal artery and lateral anterior arteries.

The median cephalic artery (CA) leaves the heart anteriorly. After passing through the pericardial membrane, this vessel passes forward along the dorsal body wall providing a series of small branches to the intestine, cardiac stomach and various muscles. It then turns ventrally and enters the *cor frontale.* The *cor frontale* consists of four bundles of somatic musculature which surround an expanded section of the vessel some 5–7 mm in length. The muscles of the *cor* contract rhythmically, probably more or less in phase with the heart, although this could not be definitely determined. After the *cor* the aorta bifurcates and these lateral branches enter the nerve sheath of the circumoral connectives where the vessels again bifurcate, one branch (the larger) proceeding to the cerebral ganglion, the other proceeding along the connective.

The lateral anterior arteries (LA) leave the heart lateral to the median cephalic artery. Each then proceeds along the dorsal body wall toward the eye of that side. Several minor branches reach the hepatopancreas, the anterior portions of the stomach and various anterior musculature. The lateral anterior arteries continue forward with branches to the eyestalks and antennae, then terminate in the antennules.

In a 10.06 g animal, the diameters of all arteries leaving the heart were measured as accurately as possible: dorsal abdominal, 0.10 cm; descending (2), 0.04 cm; median cephalic, 0.05 cm; antennary (2), 0.02 cm; hepatic (estimate), 0.02 cm.

Return system. A series of complex sinuses collects blood from the tissue spaces in the anterior of the animal. These sinuses merge and form two main channels directed ventro-posteriorly which merge just posterior to the esophagus. Receiving a number of small channels from the hepatopancreas visceral tissues and the anterior appendages, this channel expands laterally to become the infrabranchial sinus supplying the gills. This sinus runs along the ventral midline and connects with the periopodal blood spaces and with the blood spaces of the pleopods *via* the ventral abdominal sinus. The infrabranchial sinus gives off branches to each of the gills.

TABLE I

The heart weight and heart volume as calculated from the geometry of the heart for G. ingens.

Whole animal wet weight (grams)	Heart weight (grams)	Heart volume (ml)
9.56	0.031	0.039
9.67	0.025	0.042
10.05	0.030	0.047
10.75	0.021	0.038
10.92	0.034	0.049

The branchio-pericardial vessels carry blood from the gills to the pericardial chamber. These vessels consist of discrete channels passing from the bases of the periopods dorso-laterally along the edge of each somite of the thorax. Each branchio-pericardial vessel enters the pericardial chamber directly.

Blood enters the heart from the pericardium through two large ostia located in the sixth thoracic somite. These ostia have large flaps of tissue which close the opening during systolic contraction.

Heart volume

The calculated heart volume and the heart weight of five *G. ingens* are presented in Table I. The heart volume for a 10 g animal is 0.047 ml. The heart weight in g is somewhat less than the volume in ml. Both heart weight and volume, when expressed as a percentage of the total body wet weight, are greater than that found in other crustacea of similar size (Maynard, 1960b).

Gill surface area

The thickness of gill exoskeleton showed a mean of about 1.001 microns with a range of from 0.873 to 1.178 microns from a series of over 400 measurements. All the different parts of the gill showed the same range of thickness and no parts were obviously specialized for support.

The gill surface areas were calculated for 15 individual *G. ingens*. The smaller individuals (<1 g) have gill surface areas of from 5 to 8 cm^2/g wet body wt. Animals between 1 and 3 g showed values between 9 and 13 cm^2/g, while larger ones are between 9 and 14 cm^2/g wet body wt. Gills freshly removed from living specimens of *G. ingens* have also been examined and the distance across which respiratory gases must diffuse ranges from 1.5 to 2.5 microns (exoskeleton plus living cells).

Blood pressure relationships

The blood pressures in the heart, pericardium, arterial system and infrabranchial sinus of *G. ingens* during normal cardiac cycling at high O_2 levels are shown in Figure 2. This figure is a summary of the pressures measured in fifteen individuals ranging in weight from 10.23 to 17.14 g wet wt. The range of pressures measured is not great. The maximum intracardiac systolic pressure was between 27 and

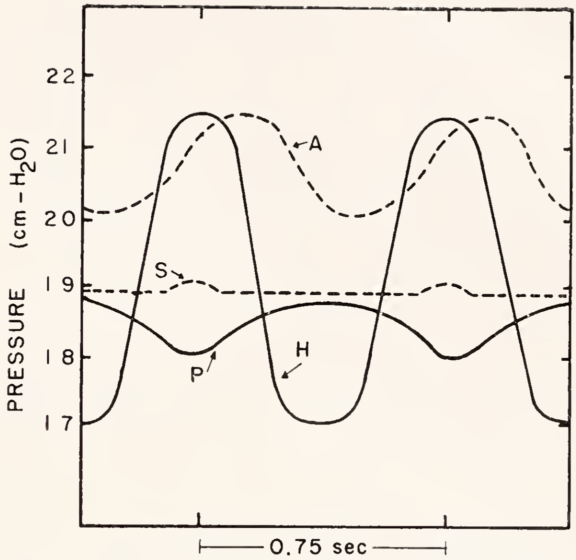

FIGURE 2. Graphical representation of normal circulatory pressures in *Gnathophausia ingens* from various regions during two systolic ventricular contractions; compiled from data obtained from 15 individuals. Heart is represented by (H); dorsal abdominal artery, (A); pericardium, (P); infrabranchial sinus, (S).

20.5 cm H_2O with a mean of 24 cm H_2O. Diastolic pressures ranged from 21.0 cm H_2O to 16.0 cm H_2O with a mean of 19 cm H_2O. The pulse pressure was between 3–6 cm H_2O with a mean of 4.5 cm H_2O. Arterial pressures (measured in the dorsal abdominal artery and in the median cephalic artery) are equal to that of the heart at systole but fall only 1–2 cm H_2O during diastolic filling. These arteries are apparently quite elastic as they expand in accepting the "pulse" of blood ejected from the heart at systole and then return slowly to the original size. The maintenance of a high arterial pressure at diastole is thus possible (Windkessel effect; Burton, 1972). There is no significant correlation between wet weight and either mean pulse pressure or maximum systolic pressure over the range of sizes of *G. ingens* examined ($P < 0.5$; t-test).

Pericardial pressure decreases about 1 cm H_2O as the systolic heart pressure rises (Fig. 2). The pressures measured in the pericardium vary somewhat along the longitudinal axis of the chamber but average slightly greater than diastolic pressure. At the ostial apertures the pericardial pressure drops to its lowest value (equal diastolic) as the valves open when filling begins.

Infrabranchial sinus pressures are greater than diastolic heart pressures by about 1.5 cm H_2O. In Figure 3, pressures recorded in the infrabranchial sinus in the region of the fifth periopod are compared with pressures measured simultaneously in the heart. In this case, the pressure drop between artery (systolic pressure) and sinus is about 3 cm H_2O. This gives a reasonable measure of tissue resistance to blood flow. The resistance to blood flow across the gills is approximately the difference between infrabranchial sinus pressure (pre-branchial) and the

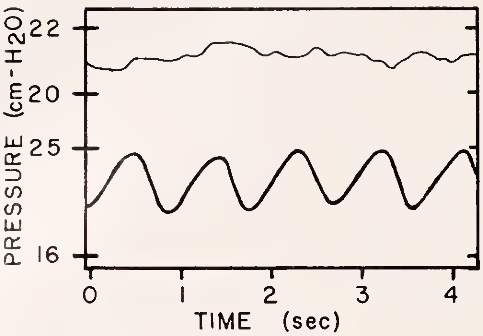

FIGURE 3. Synchronous recordings of blood pressure in *Gnathophausia ingens* in the infrabranchial sinus (upper trace) and the heart (lower trace). These are tracings of the original records which were made on an oscillograph and could not be reproduced properly for publication.

minimum diastolic pressure (post-branchial). This value averages about 2 cm H_2O pulse pressure.

Blood pressure measurements were also made on animals under sustained conditions of low oxygen such as occur in the oxygen minimum layer; *e. g.*, 0.5 ml O_2/l. Systolic and diastolic heart pressures, averaged for four animals (9.8, 10.5,

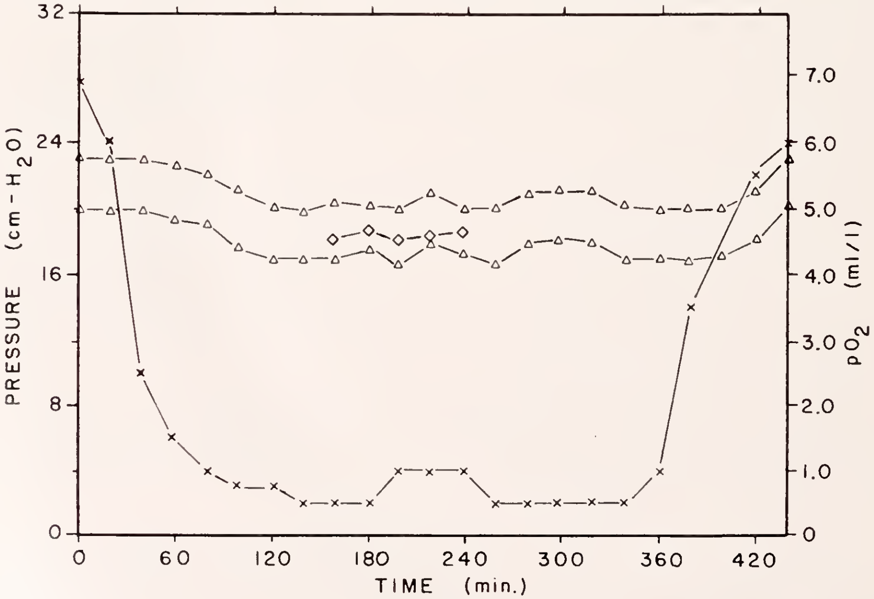

FIGURE 4. Summary of blood pressure changes occuring in four *Gnathophausia ingens* during exposure to low oxygen. Pressures measured in the heart are shown by triangles; upper line, systolic; lower line, diastolic. Diamonds show pressure measured in the infrabranchial sinus of two animals. The oxygen concentration during the experiment is shown by the symbol X.

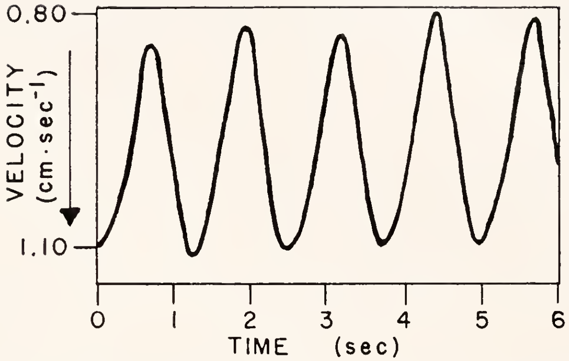

FIGURE 5. Record of blood velocity in the heart of a 10.56 g (wet weight) *Gnathophausia ingens* at low oxygen (0.50 ml O_2/l). This is a tracing of the original record which was made on an oscillograph and could not be reproduced properly for publication.

11.37, and 13.54 g wet wt), are plotted with the pO_2 against the time of the experiment in Figure 4. The range of values measured was not great; for example, the systolic range at high oxygen was 24.8 to 22.3 cm H_2O. As is shown in Figure 4, the pressures in the heart drop about 3 cm H_2O as the pO_2 drops below about 1.5 ml/l and remain within 1.0 cm H_2O of the lower value for the duration of the exposure to low oxygen. When the pO_2 is again elevated, the blood pressure returns to the high O_2 value. During the low oxygen portions of these experiments, the infrabranchial sinus pressures were measured in two animals. These pressures are plotted in Figure 4 also. The 13% drop in blood pressure at low oxygen is correlated with both a decrease in heart rate and a decrease in blood velocity in the heart (see below).

Blood velocity

The heart of *G. ingens* is a more or less tubular structure and each systolic contraction apparently moves a "pulse" of blood along its longitudinal axis (see anatomy section, above). For measurements of the blood velocity in the heart, the flow-probe was placed in the lumen of the heart midway between the posterior valve and the ostial openings (see Fig. 1). Heart-blood velocity measurements are, therefore, a measure of the velocity of the posteriorly-proceeding "pulse." A typical recording (10.56 g animal) made in this way is shown in Figure 5. At a heart rate of 48 beats/min, the maximum velocity reached is 1.10 cm/sec; the minimum velocity is 0.80 cm/sec.

The interpretation of velocity records such as that in Figure 5 is complicated by the fact that the flow-probe does not discriminate between turbulent and laminar flow and responds equally to flow in any direction. Using records of blood velocity pulses and blood pressure pulses obtained simultaneously in the heart of the same animal, we have ascertained that blood velocity records from the heart of *G. ingens* show the following. First, there is a marked increase in velocity during the systolic contraction with the peak velocity occurring just before the end of systole (about 20–30 milliseconds in the case shown in Fig. 5). This is followed by a decrease in

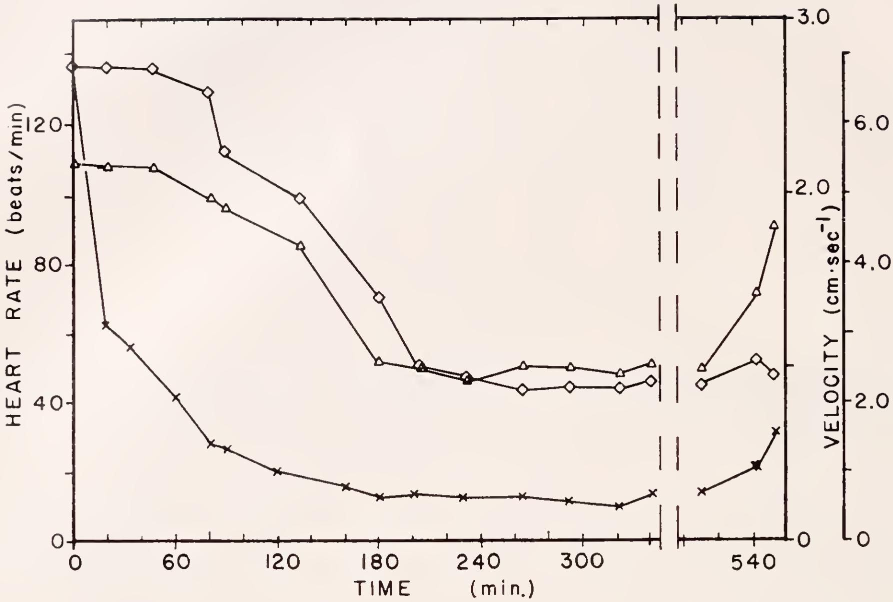

FIGURE 6. Effects of sustained low oxygen conditions on heart rate and blood velocity in a 9.38 g (wet weight) *Gnathophausia ingens*. The heart rate is shown by triangles, the blood velocity by diamonds and the oxygen concentrations by X.

velocity during the relaxation and filling phase of the cardiac cycle with the minimum velocity occurring well before the beginning of systole (about 70–100 milliseconds in Fig. 5). To derive a single value for the velocity of blood moving in the heart during systole, we have taken a typical one-minute series of contractions, calculated a representative value for the systolic portion of the cycle for each pulse and then a mean value for all the pulses occurring in that minute. To determine a representative velocity for a single systole, we have taken the mean velocity of the highest (most rapid) 75 per cent of the acceleratory portion of the velocity curve. A more sophisticated method, integration of that portion of each velocity curve from beginning to end of systole, yielded results that were within five per cent of this estimate. Since a large amount of data was involved, we chose to use the simpler method. In the case shown in Figure 5, the mean systolic velocity is 0.99 cm/sec.

The flow-probe was also introduced into the heart anterior to the ostial openings. The blood velocity measured in this region is similar in magnitude to that measured in the posterior of the heart. The recordings made here were generally inconsistent and the heart often ceased beating within a few minutes following introduction of the flow-probe. In what follows, heart-blood velocity refers only to records obtained from the posterior region of the heart.

Eight experiments were conducted in which the velocity of blood moving in the heart was measured under different oxygen tensions and under sustained low oxygen such as that occurring in the oxygen minimum layer (0.5 ml O_2/l). The results of one of these experiments (9.38 g animal), which we consider representative of the majority, are shown in Figure 6. Here the mean systolic velocity, heart rate and oxygen tension are plotted against time. Above about 2.5 ml O_2/l the contraction frequency was apparently independent of external oxygen. As the pO_2 dropped further, the heart rate fell from over 100 beats/min to 50 beats/min or less as the oxygen level reached 0.5 ml O_2/l. The heart rate then remained within a few beats/min of the lower rate for the duration of the low-oxygen exposure.

In each of the experiments in this series, the mean systolic velocity varied with the heart rate as the pO_2 changed. In the experiment shown in Figure 6, the mean systolic velocity at high heart rates (over 100 beats/min) is 2.71 cm/sec. As the heart rate drops, the velocity drops as well, so that at low oxygen levels (0.5 ml O_2/l), where the heart rate is about 42 beats/min, the mean systolic velocity is 0.95 cm/sec. Like the heart rate, the velocity increases as the pO_2 is increased near the end of the experiment. In all of these experiments at higher oxygen levels (above 1.5 ml O_2/l), the heart rate varied from 120 to 86 beats/min, while the mean velocity varied from 4.71 to 2.35 cm/sec. At low oxygen levels (0.5 ml O_2/l), the heart rate varied from 48 to 36 beats/min, while the mean systolic velocity ranged from 1.65 to 0.54 cm/sec. The systolic contraction is of longer duration at lower heart rates. In several recordings the speed of recording the output from the flow-probe was increased so that the relative proportions of each beat due to systole and diastole could be determined. At heart rates of 118 beats/min, the duration of systolic contraction is about 0.1 sec or 20 per cent of the stroke time. At heart rates under low oxygen conditions (48 beats/min), the duration of systolic contraction is about 0.5 sec or 40 per cent of the stroke time (see Fig. 5).

The measurements of blood velocity in the heart of *G. ingens* are of interest primarily as a means of assessing possible changes in the cardiac pulse in response to changing external, and by implication, internal, oxygen levels. Of greater significance physiologically, and particularly useful for making interspecific comparisons of circulatory parameters, are measurements of arterial blood velocity. In two large specimens of *G. ingens* (13.4 and 14.7 g wet wt), blood velocities were measured in the dorsal abdominal artery, 0.5 cm posterior to the heart. The flow-probe was introduced into the vessel after inserting it through the articular membrane between the second and third abdominal somite. As the diameter of the artery at the point of measurement was about 0.1 cm, the flow-probe seemed to interrupt the flow to some extent by its presence and the velocity recordings proved difficult to interpret in most instances. Such data as were obtained suggested that arterial velocities of from 1 to 2 cm/sec occurred under low oxygen conditions and from 1.5 to 4 cm/sec under high oxygen conditions (above 1.5 ml O_2/l).

Attempts were also made to measure blood velocity in the infrabranchial sinus to compare with the arterial measurements. This sinus, at its largest dimension, consists of a wide, very thin chamber and the flow-probe surface could not be positioned free of tissue. While this resulted in erratic recordings, the results obtained suggest that flow in this sinus is fairly rapid, perhaps approaching to 3–4 cm/sec in an 11.45 g *G. ingens.*

The decreased heart rate, mean systolic velocity and arterial velocity under low oxygen conditions suggest that the output of blood from the heart (stroke volume) is significantly reduced. In order to determine if this is so, and hopefully to gain some idea of the magnitude of this reduction in flow, changes in the velocity of blood flowing through the hepatopancreas in response to lowering of the external oxygen

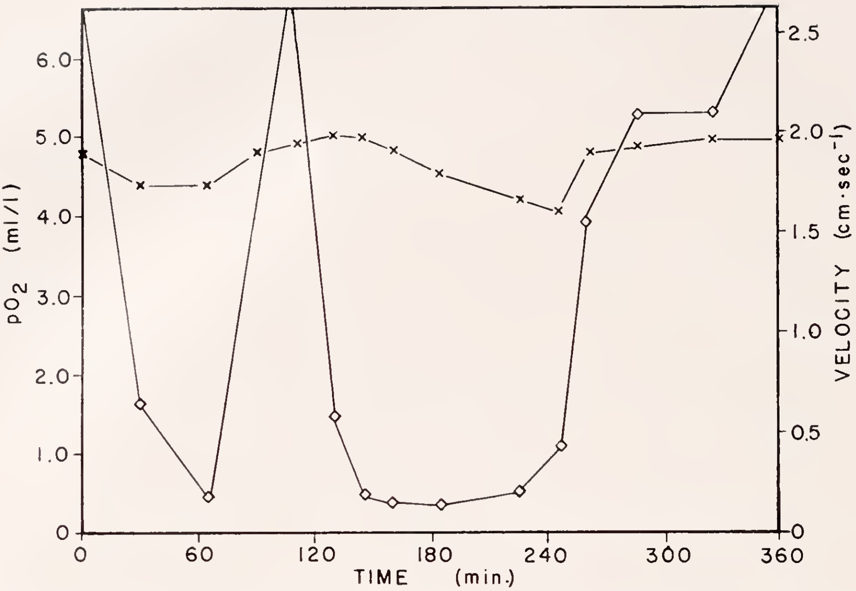

FIGURE 7. The effect of low oxygen on blood velocity in the hepatopancreas of a 10.93 g (wet weight) *Gnathophausia ingens*. Blood velocity is shown by the symbol X and oxygen concentration by diamonds.

tension were measured in three specimens of *G. ingens*. The hepatopancreas in this species is extremely large, occupying almost the entire (approximately 70%) interior of the animal with other tissues located next to the exoskeleton.

The blood velocity and pO$_2$ are plotted for the duration of an experiment with a 10.93 g animal in Figure 7. Since the flow-probe tip was in contact with tissue, the absolute velocity recorded in this experiment is probably incorrect. However, the relative change in the velocity of blood flowing through this organ as the pO$_2$ decreases is of particular interest. In this case, the velocity decreases by no more than 16 per cent after 110 minutes of oxygen less than 1.0 ml O$_2$/l. In the other two such experiments, the decrease in blood velocity in the hepatopancreas was similar: 11.45 g animal, 21%; 12.02 g animal, 14%. Assuming that the physical dimensions of the circulatory channels within the hepatopancreas do not change, it appears that the decline in cardiac contraction frequency and mean systolic blood velocity are paralleled by a decrease in blood flow through this organ. The absolute decrease in volumetric flow is, due to the complexity of the arterial supply to the hepatopancreas, impossible to calculate. However, again assuming that the dimensions of the blood channels do not change, a reduction in blood velocity of from 14–21% would mean a reduction in total flow of the same relative magnitude.

Heart rate and EKG

Data on heart rate at different oxygen levels can, of course, be derived from either the pressure-pulses or the blood velocity pulses recorded in those particular experiments. To determine whether or not the physical presence of these monitoring devices affected the rate of contraction, EKG recordings were made both with and without the pressure-needle or flow-probe in place. The results of these experiments showed that the shape of the EKG and the expected frequency of contraction were apparently unaffected. Due to rather high noise levels in the EKG records, no further use was made of these data, all heart rate information being derived from either the pressure pulses or the blood-velocity pulses.

All of the heart rate information obtained in this study was combined and plotted against oxygen tension. At oxygen concentrations above 1.5 ml/l, the rate is variable but averages 110 beats/min (range: 128–78). At low oxygen concentrations (less than 1.0 ml/l), the average is 42 beats/min (range: 86–30). The decline in heart rate to a steady, low rate does not follow the drop in pO_2 (below about 2.0 ml O_2/l) directly but lags by several minutes. This lag in cardiac response to diminished external oxygen suggests that the oxygen-sensitive mechanism is internal and that an excess of oxygen remains in the blood (or tissues) for some time beyond the external decrease.

DISCUSSION

In oxygen minimum layer conditions such as occur off the coast of Southern California, populations of *Gnathophausia ingens* live aerobically at oxygen concentrations as low as 0.20 ml/l (Childress, 1968, 1969, 1971). The youngest and oldest individuals may live at somewhat higher oxygen concentrations (Childress, 1969); however, the individuals studied here and previously, all fall in the intermediate size range which is characteristically found in oxygen minimum layer depths (500–900 meters). It is important to remember therefore that in evaluating our results, the "normal" oxygen environment of this species is between 0.20 and 1.0 ml O_2/l. This is in contrast to previously studied species which either live continuously near oxygen saturation or live in intertidal zones where periodic low oxygen stress is dealt with by air breathing, anaerobiosis or behavioral modifications. For these reasons, in the following discussion, we will generally compare the performance of *G. ingens* at 0.50 ml O_2/l with that of other species near air saturation.

Gnathophausia ingens is remarkably effective at extracting sufficient oxygen for routine metabolism from water at low partial pressures. At the organismal level this effectiveness is the result of the combination of three factors. First, *G. ingens* maintains an exceptionally high ventilation volume (up to 8 body volumes/min; Childress, 1971). This is seen most readily when this species is compared to other water breathers. The ratio of ventilation volume to oxygen consumption ($\dot{V}_{O_2}$) in *G. ingens* is about 7000 ml H_2O/ml O_2 STPD. The same ratio is about 800 for octopus, 350 for crayfish (Dejours, Garey and Rahn, 1970) and 1200 for *Cancer magister* (Johansen, Lenfant and Mecklenburg, 1970). Secondly, this species has a relatively low rate of oxygen consumption (0.034 ml/g/hr; Childress, 1971). Thirdly, *G. ingens* shows a very high percentage utilization of oxygen (50–80%; Childress, 1971).

As we have not examined the mechanisms involved in maintaining a high ventilation volume, we will not deal with the first factor in this discussion. The second factor, low $\dot{V}_{O_2}$, has been discussed by Childress (1975). We are concerned here with the mechanism(s) underlying the maintenance of a high per cent utilization at high ventilation volumes and environmental partial pressures as low as

6 mm Hg O_2. The major problem is how this animal can transport relatively large amounts of oxygen (0.034 ml/g/hr) from the exterior to the mitochondria with such a low oxygen partial pressure gradient (maximum of 6 mm Hg at external partial pressure of 6 mm Hg). The critical points in this transport are: a) the first diffusion step, inhaled water to blood; b) the circulation of blood from the gills to the tissues; and c) the second diffusion step, blood to mitochondrion. Adaptation to deal with a low gradient might occur at any of these levels. We would not expect extensive adaptations at the mitochondrial level since mitochondria apparently function at very low oxygen partial pressures generally. We have sought answers to the central question in the first diffusion step (water to blood) and in the circulation.

At the first diffusion step the critical factors are: 1) the rate of delivery of water to the gill surface, 2) the partial pressure of oxygen at the gill surface, 3) the gill surface area, 4) the diffusion distance across the gills, 5) the permeation coefficient across the gills, 6) the partial pressure of the blood in the gills, and 7) the flow rate of blood through the gills.

The high per cent utilization indicates that delivery of water to the gill surface is quite effective with little dead space. The complex, foliaceous nature of the gills is undoubtedly a factor in this (Childress, 1971). Although we have made no measurements of the partial pressure in contact with the gills, a high ventilation volume and the observation that virtually all of the respiratory water passes through the gills (Childress, 1971) suggests that the partial pressures here probably range from equal to those in the inhaled water to slightly less than those in the exhaled water.

The gill surface areas measured in *G. ingens* are larger than the areas measured for most previously studied crustaceans and fishes, and are about equal to the greatest areas measured in those other species (Gray, 1954, 1957). These other crustaceans and fishes, however, are larger and have comparable or higher $\dot{V}_{O_2}$'s than *G. ingens.* It seems apparent that one of the adaptations of *G. ingens* to increase oxygen diffusion across its gills in the presence of a low oxygen gradient is a very large gill surface area relative to its $\dot{V}_{O_2}$. In Table II we have compiled data from the literature on the oxygen consumption rates and gill surface areas for a variety of species of fishes and crustaceans and computed the ratio. Table II shows that the O_2 uptake per unit area of gill ($\dot{V}_{O_2}/A$) in *G. ingens* is much less than in all fishes examined and lower than all crabs except the largest and most sluggish.

Another aspect of the gill surface data is the suggestion that small specimens of *G. ingens* have appreciably less gill surface area per unit wet weight. This is different from the usual pattern in which larger animals have less gill area/unit wt (Hughes, 1927b, Gray, 1957). This may indicate that some elements of the total transport system become disproportionately less effective as the animals grow in size. However, the thickness of the gill exoskeleton remains roughly constant over the entire range of sizes studied. Another possible explanation might be that the smaller individuals rely to a greater extent on extrabranchial oxygen exchange than do the larger individuals. Childress (1971) demonstrated that the larger individuals do not use extrabranchial oxygen exchange to any measurable degree.

The diffusion distance across the gills (exoskeleton plus living cells) is about 1.5 to 2.5 microns in *G. ingens* and this is comparable to that found in fishes with the shortest distances and less than in many fishes (Hughes, 1972b). Unfortunately,

TABLE II

Ratio of oxygen consumption rate ($\dot{V}_{O_2}$) to gill surface (A) in a variety of species. Letters following $\dot{V}_{O_2}$ indicate standard (S) routine (R) or active (A) rate.

Species	Weight g	$\dot{V}_{O_2}$ ml/g/hr	° C	A cm²/g	Weight g	$\dot{V}_{O_2}$/A µl/cm²/hr
Fishes:						
Anguilla rostrata	40	(1) 0.088 (S)	17	(2) 3.02	428	29.2
Ameiurus nebulosus	~160	(3) 0.046 (S)	20	(3) 2.43	163	18.9
Chaenocephalus aceratus	500–1800	(4) 0.016 (S)	0	(5) 1.20	100–1000	13.3
Cotostomus commersoni	62– 200	(3) 0.060 (S)	20	(3) 2.85	206	21
Cyprinus carpio	~190	(3) 0.066 (S)	20	(3) 2.66	184	24.8
Mugil cephalus		(6) 0.2 (R)	24	(2) 9.54	166	21
Salmo gairdnerii	300– 600	(7) 0.027 (S)	15	(8) 2.40	100	8.9
Scyliorchinus stellatus		(9) 0.038 (S)		(10) 2.60	100	14.6
Tinca tinca	30	(1) 0.067 (S)	18	(9) 3.83	140	17.5
Crustaceans:						
Callincctes sapidus		(11) 0.68 (R)	27	(11) 13.7	143	5
Carcinus maenas		(12) 0.094 (R)		(12 7.8		12.2
Gnathophausia ingens	5–10	(13) 0.017 (S)	5.5	(14) 10.0	5–10	1.7
	5–10	(13) 0.034 (R)	5.5	(14) 10.0	5–10	3.4
	5–10	(13) 0.151 (A)	5.5	(14) 10.0	5–10	15.1
Libinia dubia		(11) 0.025 (R)	27	(11) 13.7	143	5
Menippe mercenaria		(11) 0.031 (R)	27	(11) 8.9	163	3.5
Panopeus herbstii		(11) 0.056 (R)	27	(11) 8.7	19.2	6.4
Uca pugilator		(1) 0.10 (S)		(11) 6.2	2.3	9.6
Uca pugnax		(1) 0.06 (S)		(11) 7.7	2.1	1.3

(1) Prosser (1973), (2) Gray (1954), (3) Saunders (1962), (4) Holeton (1970), (5) Hughes (1972a), (6) Nicol (1967), (7) Holeton (1971), (8) Hughes (1972b), (9) Hughes (1970), (10) Baumgarten-Schumann and Piiper (1968), (11) Gray (1957), (12) Hughes, Knights and Scammell (1969), (13) Childress (1971), (14) this study.

there are as yet no measurements of this diffusion distance for other crustaceans. However, one can make a rough comparison of the functioning of the gills of specimens of *G. ingens* and those of other crustaceans and fishes by using the concept of diffusing capacity (Hughes, 1972b). This parameter (D, ml O_2/min/kg wet wt/mm Hg) describes the amount of oxygen transferred across the gills of an animal for each 1 mm Hg O_2 of gradient. It may be estimated from measurements of gill area, blood-water diffusion distance and the permeation coefficient in which case it is referred to as $D_{\dagger}$. It may also be estimated from the $\dot{V}_{O_2}$ and the diffusion gradient across the gills (ΔP_G) in which case it is known as D_g. Hughes (1972b) says that in general, D_g is about one tenth of $D_{\dagger}$ due to factors not accounted for in these calculations. Since information is available for $\dot{V}_{O_2}$ and ΔP_G in two crustacean species, it is possible to calculate D_g for them and compare it with the $D_{\dagger}$ for *G. ingens*. These comparisons are shown in Table III where we have listed diffusing capacities for a number of species of fishes and crustaceans. Calculations in this table are based on the equation $\dot{V}_{O_2} = (K \cdot A/P_{O_2})/t$ from Hughes (1972b). K is the Krogh permeation coefficient expressed as ml/min/cm²/µ/mm Hg and taken as $1.71/10^{-4}$ for crustaceans and $1.45/10^{-4}$ (Krogh, 1941) for fishes and corrected for temperature (2%/° C, Randall, 1970). A is the gill surface in cm²/kg; t is the water-blood distance in µ; and $\dot{V}_{O_2}$ is expressed in ml O_2/kg/min. $D_{\dagger}$ for

TABLE III

Diffusing capacities (D_g = transfer factor) for a number of species of fishes and crustaceans and calculated diffusing capacities (D_t) of their water-blood barriers. Either D_g or D_t figures in parentheses are estimated values assuming $D_t = 10\ D_g$. The lower ΔP_g values in parentheses have been estimated from the relationship $\Delta P_g = \dot{V}_{O_2}/D_t$ while the higher values have been estimated from $\Delta P_g = \dot{V}_{O_2}/10/D_t$. [Table adapted from Hughes (1972b)].

Species	Gill area cm²/g	Wet weight g	Water-blood barrier (t) μ	Diffusing capacity (ml/min/kg/mm Hg)		ΔP_g mm Hg
				Calculated D_t	Measured D_g	
Fishes:						
Salmo gairdnerii (1)	2.40	100	6	0.050	(2) 0.0080	56
Scyliorhuinus stellatus (3)	2.60	100	11	0.0272	(4) 0.0087	73
Tinca tinca (3)	2.50	100	3	0.0525	(1) 0.007	77
Chaenocephalus aceratus (5)	1.20	100	6	0.030	(6) 0.0043	63
Ameiurus nebulosus (7)	1.50	100	10	0.023	(8) 0.0015 =	(9) (33–520)
Scomber scombrus (10)	11.58	182	(1) 1.2	1.3	(0.13)	
Trachurus trachurus (10)	10.74	125	(1) 2.22	0.70	(0.071)	
Pleuronectes platessa (10)	4.33	86	(1) 3.85	0.17	(0.017)	
Crustaceans:						
Cancer magister (11)		1000		(0.09)	0.0086	60.5
Panulirus interruptus (12, 13)		600		(0.08)	0.0077	140
Gnathophausia ingens (14)	10.0	10	2.0	0.60	(0.06)	(0.373–3.73)

(1) Hughes (1972b), (2) Randall, Holeton and Stevens (1967), (3) Hughes (1970), (4) Baumgarten-Schumann and Piiper (1968), (5) Hughes (1972a), (6) Holeton (1970), (7) Steen and Berg (1966), (8) Fisher, Coburn and Forster (1969), (9) calculated using $\dot{V}_{O_2}$ from Saunders (1962), (10) Gray (1954), (11) Johansen, Lenfant and Mecklenburg (1970), (12) Redmond (1955), (13) Winget (1969), (14) Childress (1971) and this study.

G. ingens is about 100 times greater than D_g for *Cancer magister* and *Panulirus interruptus*, suggesting that the diffusing capacity of the gills of *G. ingens* is considerably greater than that of either of these animals. If one makes the further approximations shown in Table III, the ΔP_g of *G. ingens* can be estimated roughly at about 4 mm Hg. The interesting thing about this estimate is that it falls within the possible range for this animal at 6 mm Hg external O_2 and is much smaller than the measured ΔP_g of other species of crustaceans and fishes. It appears then that *G. ingens* achieves a high diffusion capacity by having a large gill surface relative to its $\dot{V}_{O_2}$ (Table II) as compared to fishes. Compared to other crustaceans, it seems that the higher diffusion capacity is either a function of a much thinner blood-water diffusion barrier or a much lower physiological dead space in the gills of *G. ingens*. During the evolution of this species to survive under low oxygen conditions, it appears to have developed the structures and functions so far considered to levels near the limits of effectiveness in oxygen transport found in aquatic animals.

Our observations concerning the circulatory system of *G. ingens* indicate that this system has been evolved for relatively high rates of blood flow. Among the modifications which favor high flow rates in this species are quantitative increases in size and capacity of circulatory system components. For example, the blood system of *G. ingens* follows the typical mysid and decapod crustacean pattern.

TABLE IV

Arterial diameters in three crustaceans.

Species	Wet weight (g)	Diameter of median cephalic artery (cm)	Diameter of dorsal- or posterior-abdominal artery (cm)	Reference
Gnathophausia ingens	12	0.050	0.100	This study
Pachygrapsus crassipes	20	0.042	0.065	Seaton (1971)
Panulirus interruptus	622	0.110	0.250	Belman (1975)

However, the arterial system in *G. ingens* appears proportionately quite large when the diameters of the main arteries exiting the heart are compared to those of corresponding arteries in lobsters and crabs as we have done in Table IV.

The single chamber tubular heart of *G. ingens* appears to be structurally similar to that of other mysids (Gadzikiewicz, 1905). However, the heart in this species is apparently relatively large for a crustacean of its size. This conclusion is based on a comparison of the data in Table I (10 g *G. ingens* heart weight = 2.8×10^{-2} g) with the predicted heart weight in a 10 g crustacean (1×10^{-2} g, from regression of heart weight on body weight for tropical decapods, Maynard, 1960b).

Since in the evolution of crustaceans there is a tendency for each vessel to supply a particular organ or organ system and to evolve to a size suitable to the needs of that region of the organism (Maynard, 1960a), we can also draw certain conclusions about blood flow from the relative diameters of vessels leaving the heart. Both the median cephalic artery and the posterior abdominal artery are disproportionately large in *G. ingens* compared to other crustaceans on a weight basis (Table IV) and compared to other vessels exiting the heart in this species (results section, this paper). The median cephalic artery also has a *cor frontale* located in such a position that it can assist in overcoming the resistance to flow imposed by the fine arterial divisions feeding into the cerebral and associated ganglia. These apparent priorities in the circulatory system agree with what we know from other points of view. That is, *G. ingens* has a well developed nervous system which explains the need for the median cephalic artery development. The development of the posterior abdominal artery is almost certainly related to the fact that this species has a considerable scope for activity which is largely accounted for by movement of the abdominal pleopods which account for about 50% of the O_2 consumption at "normal" levels of activity and much larger percentages at higher levels (Childress, 1968, 1971). This information concerning the anatomy of the circulatory system of *G. ingens* supports the suggestion that this species may have high blood flow rates.

The blood pressure data for this mysid also indicate circulatory modifications of adaptive significance. While absolute blood pressures over 20 cm H_2O in *G. ingens* may appear to be in conflict with the general characterization of crustacean blood systems as operating at low pressures (Prosser, 1973), other work in our laboratory indicates that the pressures observed in *G. ingens* are comparable to those found in a variety of littoral crustaceans (Belman, unpublished). The recordings of blood pressure (summarized in Fig. 2) suggest that the circulation in *G. ingens* is primarily heart driven and that body movements play only a limited role. The pattern of pressure in *G. ingens* differs from that in other crustaceans in that about 40 per cent of the peripheral resistance occurs across the gills in this species, while in other crustacean species investigated (Burger and Smythe, 1953; Blatchford, 1971; Belman, 1975) less than 20 per cent of the peripheral resistance occurs across the gills. This difference may be the result of either longer or finer blood channels in the gills of *G. ingens* or in a relative reduction in the resistance of other parts of the circulatory system. In either case, *G. ingens* puts a greater fraction of its cardiac work into movement of blood through its gills than do crustaceans from higher oxygen habitats.

The data on heart rate in *G. ingens* at different oxygen partial pressures are similar to those of crustacean species whose normoxic environment is near air saturation, showing comparable rates and lowered heart rates at lower environmental oxygen partial pressures (Larimer, 1962, 1964; Larimer and Gold, 1961; Stiffler and Pritchard, 1972). The suggestion for these other species has been that the lower heart rates are not an adaptive response but a result of oxygen stress on the heart near or below the lower critical oxygen partial pressure (P_c). The data on *G. ingens* are difficult to understand in this light, since this animal regulates its oxygen consumption down to very low partial pressures and its normoxic condition is one of low oxygen. Therefore, the heart rate of *G. ingens* is at least not interfering with this regulation. There are at least two possible explanations for this phenomenon in *G. ingens*. First, the high heart rates at high oxygen partial pressures may be a result of excitement of the animal and therefore do not indicate what the "normal" rates would be. Secondly, the heart rate may not actually be a good indication of volume flow of blood which is the physiologically important parameter. The first of these may well be an important factor in these observations since the experiments lasted only a few hours and the animals generally displayed a markedly high level of pleopod activity. This could result in $\dot{V}_{O_2}$ as much as 4.5 times greater than the routine rate and since, as Childress (1968, 1971) has shown, P_c increases as a function of increasing $\dot{V}_{O_2}$, the P_c could go as high as 25 mm Hg O_2 (about 1.25 ml O_2/l). It seems apparent that our data (Figs. 4, 6, and 7) describe the maximum heart rate, blood pressure, and blood flow which the animal maintains at the maximum $\dot{V}_{O_2}$ which it can sustain at a given P_{O_2}. This is supported by the fact that the major drop in heart rate occurs at about the P_c for a maximally active animal.

The second possible explanation for the decline in rate with declining P_{O_2} is supported by two parts of our findings which suggest that blood flow may decline more slowly than does heart rate. The first is the observation that the decrease in heartbeat frequence is coincident with an almost fivefold increase in the duration of the systolic contraction. The lower rate of spread of the contractile event from its origin to its terminus may allow more complete emptying of the heart, increasing the effective stroke volume of the heart. Due to this, minute volume might decrease proportionately less than heart rate. The second piece of data supporting the suggestion that volumetric flow declines less than heart rate is the data on blood flow in the hepatopancreas (Fig. 7). In the animals examined, apparent blood velocity decreased about 17% over the range where heart rate would be expected to decline about 60%.

The recordings of blood velocity in the intact circulation system of *G. ingens* are, to our knowledge, the first direct measurements made in such a small crustacean. The mean velocity of blood in the heart of a 10 g *G. ingens* would be about 2.7

cm/sec at high oxygen and 1.0 cm/sec at low oxygen (Fig. 6). The velocity in the posterior abdominal artery is on the order of 1.5 to 4 cm/sec under higher O_2 and about 1 to 2 cm/sec at low O_2 levels. The flow in the infrabranchial sinus may approach 3 to 4 cm/sec. These velocities range from about the same as to about one-half those found in 700 g lobsters (*Panulirus interruptus,* Belman, 1975). The velocities in *G. ingens* seem remarkably high when considered relative to the small size of this species and to the relatively large size of the blood vessels leaving its heart. For the reasons discussed above, these measurements probably represent maximum values at particular oxygen partial pressures.

The most important parameters of circulation concern the volume pumped in relation to time (minute volume) and in relation to total blood volume (turnover time). These can be estimated in a variety of ways as described below. The simplest method of estimating cardiac output is to assume that heart volume equals stroke volume and to multiply this by the heart rate in beats per minute. The minute volume calculated by this method for a 10 g *G. ingens* ranges from 197 ml/kg/min (0·5 ml O_2/l, 42 beats/min, heart volume = 0.047 ml) to 517 ml/kg/min (greater than 1.5 ml O_2/l, 110 beats/min). These values represent the absolute maximum rates which this system could produce. Since stroke volume is certainly somewhat less than the heart volume, higher estimates must have to be in error and actual values would be expected to be lower.

The data presented here on blood velocities provide another method of estimating cardiac output. By the Poiseuille-Hagen equation, $F = P/\pi/8/1/n/R^4/L$, total blood flow (F) in a given length of blood vessel (L) is proportional to the pressure drop (P), inversely proportional to the absolute viscosity (n), and proportional to the fourth power of the radius (R) (Ganong, 1969). Knowing the flow (F) in one of the vessels (assuming an equal pressure drop in all) using this relation, since the viscosity, pressure and length factors will all be small relative to the size factor (R) and can be presumed constant for the argument. It follows (equation 1) that $F = K/R^4$, where F is flow in cm^3/sec, K is a constant and R the radius in cm. A value for K can be derived from total flow (F) in the dorsal abdominal artery. In a 12.2 g *G. ingens* the velocity of blood in the dorsal abdominal artery was observed to be 1.5 cm/sec at low P_{O_2} and the arterial radius at the point of measurement was 0.05 cm (area = 0.008 cm^2). The flow in this artery is the product of the area times the velocity: 0.12 cm^3/sec or 0.72 ml/min. It follows (equation 2) that $K = F/R^4 = (0.012)/(0.05)^4 = 1.92/10^3$. Using the values given in the results for the diameter of the vessels leaving the heart of a 10.06 g individual and K from equation (2), the flow in each artery was calculated by equation (1). The sum of these flows is an approximation of the minute volume (0.805 ml/min). The corresponding cardiac output is 80 ml/kg/min. The full range of flows from the highest measured at high P_{O_2} to the lowest measured at low P_{O_2} is from about 225 ml/kg/min to 55 ml/kg/min. These values are possible since they are lower than the maximum values estimated above and when they are compared with the maximum values suggest that stroke volume is equal to about 25 to 50% of heart volume.

One can also estimate minute volume from the Fick principle if one knows the pre- and postbranchial blood O_2 concentrations and the $\dot{V}_{O_2}$. While we lack measurements of blood (O_2), we can make an estimate based on the $\dot{V}_{O_2}$ of this species at 6 mm Hg environmental oxygen concentration (0.56 ml O_2/kg/min, Childress, 1971), assuming that the solubility of oxygen in the blood is the same as in sea water (no respiratory pigment). Using such assumptions one arrives at an estimate of a minute volume of 0.02 l/min for a 10 g *G. ingens.* This is impossibly high, exceeding the maximum possible value by fourfold. It suggests that this animal must have a respiratory pigment and that the capacity of blood for O_2 at 6 mm H_g O_2 must be at least 9 to 36 times greater than sea water (that is, between 1.9 and 7.8 ml O_2/l), depending on the actual blood flow rates.

An accurate Fick principle estimate will have to wait until the internal oxygen concentrations of this animal can be measured. Of the other two estimates, those based on heart volume suggest that this species has the potential for high blood flow rates, while the values based on measured flow rates indicate that this animal does indeed realize rather high minute volumes. For comparison with the cardiac output values of 55 to 225 ml/kg/l in *G. ingens* we can examine the lobster *Panulirus interruptus* which has cardiac output estimates between 80 and 150 ml/kg/min for a 700 g animal (Belman, 1975; *P. interruptus* is especially good for comparison since the $\dot{V}_{O_2}$ of a 700 g individual at 16° C is nearly the same as that of a 10 g *G. ingens* at 5.0° C; Winget, 1969). The cardiac output of a 1.0 kg *Cancer magister* (Johansen *et al.*, 1970) is 29 ml/kg/min and for a 0.45 kg *Homarus americanus* (Burger and Smythe, 1953) it is 22 to 67 ml/kg/min. Apparently *G. ingens* is capable of maintaining blood flow greater than most crustaceans examined and about as great as the highest values estimated previously. This clearly is of value for an animal living as *G. ingens* does, at very low P_{O_2}.

A value useful in comparing circulatory dynamics is the turnover time; that is, the time required for the complete circulation of a unit volume of blood. Assuming a total blood volume of from 20 to 30% of the wet weight (a reasonable estimate for crustaceans; Maynard, 1960a), and using a cardiac output between 55 ml/kg/min and 225 ml/kg/min, the turnover time will be from 0.9 to 5 minutes in a 10 g *G. ingens.* Calculated turnover times in decapods are 2.1 min in a 0.65 kg *P. interruptus* and 10 min in a 1.0 kg *C. magister* (Belman, 1975); 1–8 min in a 0.45 kg *H. americanus* (Burger and Smythe, 1953); 1–5 min in a 0.22 kg *C. maenas* (Blatchford, 1971). These comparisons show that circulation in *G. ingens* is about as rapid as in those crustaceans which exhibit the most rapid blood flows.

From values for pulse pressure and minute volume one can estimate the metabolic cost of cardiac output. Assuming that heart tissue is about 15 % efficient (Burton, 1972), the cardiac work for a 10 g *G. ingens* is about $3.5/10^{-4}$ cal/ml while for a 0.6 kg *P. interruptus* it is about $2.4/10^{-3}$ cal/min. This difference in efficiency probably is the result of the proportionately larger diameter and shorter blood vessels in *G. ingens.* The higher efficiency may be of significance as an energy conserving mechanism for *G. ingens* which lives at relatively food-poor greater depths (Childress, 1975).

In conclusion, with respect to those aspects of oxygen uptake considered in this manuscript and in Childress (1971), *G. ingens* appears to possess systems which are developed to as high a degree of effectiveness as found in any aquatic animals. What makes *G. ingens* unique among those species already studied and makes possible its unusual effectiveness at removing oxygen from water is not an unusual degree of development of one system, or some sort of special mechanism, but rather the high degree of development and effectiveness of all of the mechanisms so far studied.

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SUMMARY

1. The anatomy of the blood circulatory system in *G. ingens* is similar to that in other crustacea, although proportionately both the heart and arterial channels are quite large.

2. *G. ingens* gill surface area ranges from 5–15 cm^2/g wet body weight. These gill surface areas are as large as the greatest areas measured in other, much larger crustaceans. A large gill surface area relative to a low oxygen consumption rate permits an increased oxygen diffusion across the gills despite a low oxygen gradient.

3. The generalized intra-ventricular blood pressure in *G. ingens* is 24/19 cm H_2O. Mean arterial pressure matches the systolic pressure. These pressures are comparable to those in the large brachyuran *Carcinus maenas.*

4. Blood velocities of from 0.54 to 4.71 cm/sec occur in the heart and arterial system of *G. ingens.* Under normoxic conditions ($pO_2 = 6$ mm Hg) heart rates average 42 b/m and arterial velocities average 1–2 cm/sec.

5. The circulation of blood in *G. ingens*, when compared to other crustaceans on the basis of minute volume, cardiac output and turnover time, appears to be remarkably effective. It is suggested that this effectiveness explains, in large part, the unique ability of *G. ingens* to remove large percentages of the available oxygen while moving large volumes of water over the gills.

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DELAYED NAUPLIAR DEVELOPMENT OF MEIOBENTHIC COPEPODS[1]

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As part of our continuing studies on the meiofauna of the North Inlet Estuary, Georgetown, South Carolina, U. S. A., we have been culturing several harpacticoid copepods in the laboratory. In the course of routine culture examination and flow chart analyses of previous cultures, we observed the continual appearance of stage 1–3 nauplii in a given culture for periods up to seven weeks after an isolated female hatched her eggs. This observation then spurred us to establish additional, rigorously controlled cultures specifically designed to observe the frequency of naupliar appearance, and indeed, in these controlled cultures as well, naupliar development was delayed. In all cases the cultures were set up with particular care to insure that no extraneous nauplii were introduced with the gravid female. As soon as the female became gravid again or her offspring matured to adulthood, they were removed, thus eliminating the possibility of that female or a F_1 (or later) producing the observed nauplii.

What is so striking is the delayed development of the nauplii. An original gravid female hatches all her eggs within a 48-hour period (the egg sac disappears after the hatch); thus, the continual appearance of nauplii must be due to their differential growth. If this is the case, it would prove to be a distinct ecological advantage to increase the ability of the species to extend its occupation of an area. We have encountered what appears to be the same "time-released" phenomenon in four species. Herewith we report on this phenomenon in these four species and speculate on its adaptive significance.

MATERIALS AND METHODS

Copepods were collected regularly during 1972–75 from subtidal sands and muds in the North Inlet Estuary, Georgetown, S. C. (30° 20′ N, 79° 10″ W) by scooping the upper 2–5 cm of sediment into plastic buckets. The buckets were filled to one-half their volume, capped and transported to the laboratory in air conditioned vehicles at a constant temperature. Upon returning to the laboratory those species which had a majority of ovigerous females (indicating ongoing reproduction in field) were individually sorted from the sediment using a mouth pipette and placed in culture dishes (8.3 cm diameter). In the original ("non-controlled") cultures ten mg of untreated natural sediment from the sampling site (from which all organisms except bacteria and algae had been manually removed) were placed in the culture dish and covered (5 cm) with 30 ‰ Millipore-filtered sea water. Individual ovigerous copepods were then introduced into this natural substrate. In the second

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("controlled") cultures, the ovigerous copepods were placed in sediment that had been frozen prior to use to remove residual life and the sediment was overlain with 5 cm of 30 ‰ Millipore-filtered sea water which had been heated to 70° C and conditioned at 18° C prior to use. All cultures were then placed in controlled environmental chambers with temperature and light/dark cycles the same as in the field. Salinity was maintained at 30 ‰ by the addition of distilled water to compensate for evaporation and monitored using a hand-held refractometer. Food, such as cultured benthic pennate diatoms (*Navicula directa*, *N. pelliculosa*) or natural ciliate-bacterial population, was introduced with ovigerous females; the cultures were never allowed to become food-limited.

In both the "controlled" and "non-controlled" cultures one ovigerous copepod was placed in each dish and observed daily until all her eggs had hatched. In the "non-controlled" cultures the female was allowed to remain in the culture until she again produced eggs. Since female harpacticoids are iteroparous [*i.e.*, stored sperm from one insemination is capable of fertilizing up to eight broods (Johnson & Olsen, 1948], the fact that an individual female became gravid again was expected. Upon noting internal egg development (prior to formation of the external egg sac), the female was removed from the culture; or if the female was not observed daily (*e.g.*, over a weekend) and the egg sac formed, she was in all cases removed to a new culture prior to complete development of the eggs. Furthermore, when the offspring reached the 4th–6th copepodite stage, they were removed to separate culture dishes. In the "controlled" cultures the female was removed immediately after hatching her first brood. Therefore, only the mother was present as an adult in the "non-controlled" cultures and, except during the time of hatching, an adult was never present in the "controlled" cultures.

After the female had completed hatching and was removed, the "controlled" cultures were examined daily and all nauplii enumerated as soon as they exhibited movement. "Non-controlled" cultures were examined at regular intervals not exceeding three days. The notation N_1–N_3 is used to note the appearance of nauplii because we could not distinguish between N_1, N_2, or N_3 stages in the culture dish. The only differences between N_1 and N_3 are a small size differential (about 20 μ) and the number of anal spines (see Rosenfield and Coull, 1974, for morphology of copepod developmental stages). However, we were able to distinguish N_1–N_3 from N_4–N_6 by observing naupliar movement. N_1–N_3 nauplii simply "wiggle" from grain to grain of sediment on the bottom of the culture dish, whereas N_4–N_6 nauplii actively crawl across the dish and rapidly swim up and down in the overlying water. Such differences in movement are obvious and thus make separation of the two naupliar classes a relatively simple procedure.

RESULTS

Table I and Figure 1 summarize the delayed naupliar developmental data for the "non-controlled" cultures, and Table II and Figure 2 for the "controlled" cultures for the four species: *Paronychocamptus* sp. (see Coull, 1976, for description), *Microarthridion littorale* (Poppe), *Thompsonula hyaenae* (I. C. Thompson), and *Pseudobradya pulchella* Sars. One must realize that our original cultures ("non-controlled") were designed to rear copepods from egg to adult—not to examine the timing of naupliar growth. It was not until we completed a thorough analysis of

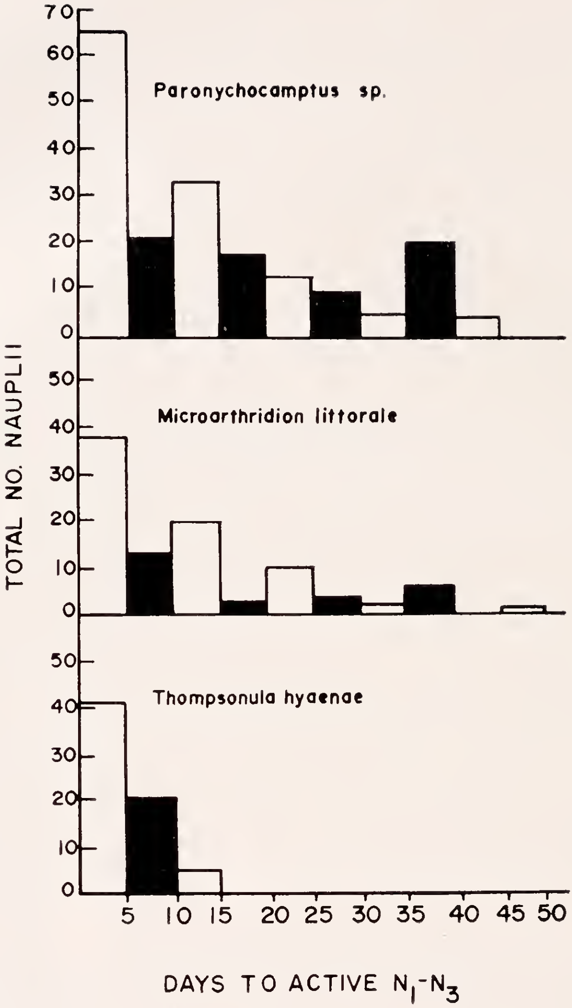

FIGURE 1. Summary data of the appearance of active stage 1–3 nauplii with time in three species of harpacticoid copepods in "non-controlled" cultures.

each individual female's offspring that we encountered the delayed naupliar development phenomenon. At this time we decided to more carefully culture the animals and examine the timing of naupliar activity. Thus the "controlled" cultures series was set up using species available in the field in adequate numbers during that season, *i.e.*, *Paronychocamptus* and *Pseudobradya*.

The data in Tables I and II are presented in a much-reduced form. We have developed flow charts of larval development for each individual but to present them would unduly clutter our report with charts not easily decipherable. One example should serve to illustrate the point. An ovigerous *Paronychocamptus* sp. (#288—

TABLE I

Summary data on naupliar development of three cultured ("non-controlled") harpacticoid copepods. Column 3 is particularly relevant in that it gives the days on which N_1–N_3 appeared in the culture and the number of N_1–N_3 on each day. Day 1 is the day of the hatch. Note that N_1–N_3 nauplii continued to appear up to 50 days (Microarthridion littorale, *#32) after the hatch.*

Species and culture number	Number of eggs	Days to N_1–N_3 appearance (and number of N_1–N_2 each day)	% mortality prior to N_1–N_3 $1 - \frac{\text{Number } N_1\text{–}N_3}{\text{Number eggs}} \times 100$
Paronychocamptus sp.			
288	20	5 (5), 8 (3), 14 (7), 28 (3), 41 (1)	5
288a	17	5 (5), 12 (3)	53
288b	17	16 (2), 36 (5)	59
288i	18	5 (1), 10 (8)	50
310a	18	5 (4)	78
310b	19	4 (5), 12 (1)	68
310c	18	6 (5), 18 (1)	67
326	20	13 (7)	65
330	18	15 (1), 31 (4), 44 (1)	67
336a	21	5 (7), 13 (5), 26 (4), 40 (4)	5
336b	20	5 (5), 36 (7)	40
339	19	4 (5)	74
347	18	2 (3), 5 (2), 21 (2), 28 (1), 42 (2)	48
348	19	16 (2), 18 (2), 20 (4)	58
349	18	2 (3), 5 (5), 18 (3), 25 (3), 36 (3)	5
347a	18	5 (4), 11 (4), 15 (2), 24 (2), 28 (1), 38 (1)	22
406	20	2 (2), 12 (5), 14 (10), 21 (1)	10
407	18	4 (2), 18 (4), 24 (2)	56
408	18	2 (8), 5 (3), 8 (2), 18 (2), 20 (1)	0
409	19	3 (6), 6 (2), 21 (1)	53
$\bar{x} \pm$ S.D.	18.6 ± 1.1		43.7
Microarthridion littorale			
32	20	2 (5), 11 (2), 25 (2), 40 (6), 50 (1)	20
32a	20	2 (3), 8 (3), 13 (1)	65
351	20	2 (4), 5 (2), 8 (3), 12 (3)	40
352	18	5 (6), 8(4), 12 (2), 20 (2)	22
353	18	2 (4), 8 (3), 12 (3), 22 (2)	33
354	18	2 (6), 12 (3), 23 (1), 31 (2)	33
355	20	5 (4), 12 (3), 22 (3), 30 (1)	45
356	20	5 (4), 12 (3), 22 (2), 30 (2)	45
$\bar{x} \pm$ S.D.	19.3 ± 1.0		37.9 ± 14.4
Thompsonula hyaenae			
27	25	4 (9), 8 (6), 10 (3), 13 (2)	20
27a	27	1 (7), 4 (12), 7 (3), 13 (3)	7
69	27	2 (13), 6 (8)	22
$\bar{x} \pm$ S.D.	26.3 ± 1.1		16.3

TABLE II

Summary data of naupliar development of two cultured ("controlled") harpacticoid copepods. Column 3 is particularly relevant in that it gives the days on which the N_1–N_3 nauplii appear in the culture and the number of N_1–N_3 each day. Day 1 is the day of the hatch.

Species and culture number	Number of eggs	Days to N_1–N_3 appearance (and number of N_1–N_3 each day)	% mortality prior to N_1–N_3 $1 - \frac{\text{Number } N_1\text{–}N_3}{\text{Number eggs}} \times 100$
Paronychocamptus sp.			
417	17	1 (3), 6 (5)	53
419	17	4 (2), 14 (1)	82
430	18	8 (1), 15 (2), 24 (2)	71
446	19	3 (14), 12 (2)	16
447	19	4 (4), 6 (6), 12 (1), 20 (1)	37
447a	18	8 (3), 12 (2), 16 (1)	67
448	19	3 (4), 12 (2)	47
449	19	4 (7), 6 (6)	32
450a	17	2 (5), 7 (1), 20 (1)	59
454	19	6 (9), 24 (1), 34 (2)	29
455	19	4 (8), 12 (4), 14 (2), 22 (2)	16
456	19	6 (8), 12 (2)	47
457	19	4 (8), 12 (4), 24 (5)	11
458	19	4 (1), 12 (2), 20 (2), 30 (1)	68
459	19	4 (8), 12 (5)	32
460	19	1 (3), 4 (8), 12 (4), 20 (2)	11
462	20	2 (2), 6 (8), 12 (2), 22 (2)	30
473	19	5 (2), 12 (4)	68
$\bar{x} \pm$ S.D.	18.6 ± 0.8		43.1
Pseudobradya pulchella			
463	15	4 (1), 10 (2), 20 (2)	67
470	16	4 (6), 15 (2), 25 (3), 30 (1)	38
472	15	8 (3), 12 (4) 20 (1)	47
475	16	3 (5), 6 (1)	63
476	15	3 (6), 6 (3), 15 (1)	33
477	15	5 (2), 15 (4), 22 (1)	54
478	15	3 (4), 6 (2), 15 (4), 26 (1)	27
479	16	3 (6), 5 (4), 15 (2), 24 (3)	7
480	15	3 (3), 5 (4), 15 (3)	33
$\bar{x} \pm$ S.D.	15.3 ± 0.5		41.0

from Table I) was isolated on 10/16/73; she hatched her 20 eggs on 10/18/73; five stage 2–3 nauplii appeared on 10/23/73; and two reached adulthood on 11/6 and 11/8/73, respectively—the others died. The two adults were then removed. On 10/26, we observed three more stage 1–3 nauplii, and one of these reached adulthood on 11/17/73. On 11/1 (14 days after the hatch in the same original culture) seven other stage 1–3 nauplii were found, three of which reached adulthood between 12/3/73 and 12/6/73. Again all were removed before copulation. On 11/15 (28 days after the hatch), three more stage 1–3 nauplii appeared, repeating the same procedure as before, and on day 41 (11/28/73) we encountered another stage 1–3 nauplii, which subsequently died. Further naupliar development was not observed after 11/28 in the original culture. *Paronychocamptus* normally carries

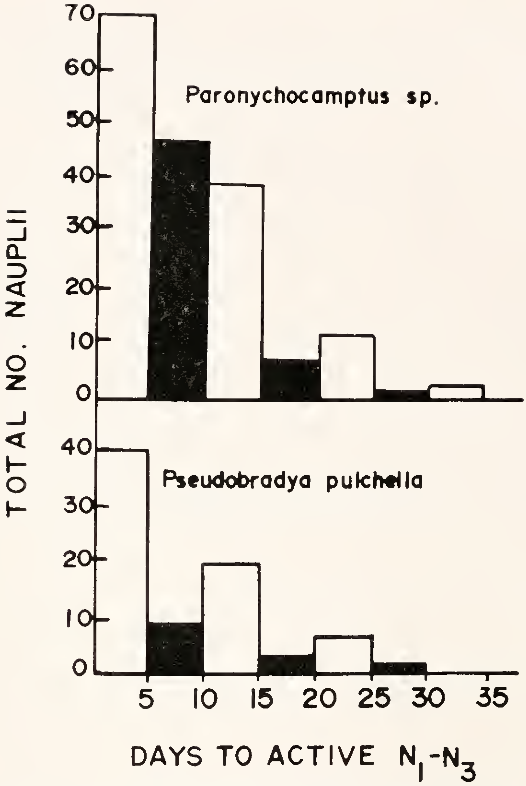

FIGURE 2. Summary data of the appearance of active stage 1–3 nauplii with time in two species of harpacticoid copepods in "controlled" cultures.

17–21 eggs (this particular individual had 20) and 19 nauplii which appeared at stage 1–3 during the 6-week period were accounted for.

The histograms, Figures 1 and 2, also illustrate that N_1–N_3 nauplii continued to appear in the cultures up to 50 days after the hatch. The greatest number of N_1–N_3 nauplii in all four species occurred in the first 1–5 days with an irregular decline in numbers subsequently. The histograms for three of the four species (*Thompsonula*, Figure 1, excepted) show secondary N_1–N_3 peaks at irregular intervals. Whether these secondary peaks are real or merely a function of small sample size is unknown. Nevertheless, the point is still made that there is obviously a prolongation of the N_1–N_3 stages in these four benthic harpacticoids. We have been relatively successful in culturing *Paronychocamptus* sp., which accounts for more data on this species. Naupliar growth is 38 (20 "non-controlled", 18 "controlled") cultures illustrates that the delayed naupliar development is a regular occurrence in this species. In fact, in every *Paronychocamptus* culture that was successfully maintained, delayed development occurred. We do not have data on as many single cultures of the other three species for two reasons: 1) successful rearing of *M. littorale*, and *T. hyaenae* was erratic, and 2) many of the earlier cultures of *Microarthridion, Thompsonula* and *Pseudobradya* were not checked frequently enough to allow for careful naupliar enumeration. Even so, delayed naupliar development is also apparent in these three species (Tables I and II, and Figures 1 and 2).

DISCUSSION

In the literature dealing with growth, culturing and development of harpacticoid copepods (*e.g.*, Johnson and Olsen, 1948; Huizinga, 1971; Betouhim-el and Kahan, 1972; Volkmann-Rocco, 1972; Rosenfield and Coull, 1974; Smol and Heip, 1974), there is only one hint of differential naupliar growth. Smol and Heip (1974) suggest delayed naupliar stages and contend that the delay is in response to increased temperature. They do not, however, give any data on the length of the individual stages and only state, "In the case of *T. discipes* it is clear that the relative duration of the naupliar stage is prolonged when the temperature increases" (Smol and Heip, 1974, p. 167).

Most of the above-mentioned studies deal with epiphytic or semipelagic forms, and particularly the relatively easily cultured *Tisbe* spp. Only Rao (1967) dealing with an interstitial copepod, *Arenopontia indica,* Muus (1967) with *Nitocra spinipes*, and Smol and Heip (1974) with *Paronychocamptus nanus* and *Tachidius discipes* have successfully cultured benthic harpacticoids; and there is some doubt as to the true substrate preference of *N. spinipes* and *T. discipes* (Muus, 1967). The non-sediment swelling forms can be cultured on large algal fragments such as *Ulva* or on a variety of other substrates (Purina Laboratory Rat Chow, Huizinga, 1971; yeast, Rosenfield and Coull, 1974). However, the true sediment-dwelling forms require a granular substrate for the organisms to grasp. Whether the mode of naupliar development reported here is unique to sediment-dwelling harpacticoids is open to speculation. Unfortunately, comparative studies are not found in the literature. However, since delayed naupliar development has not been reported in the many cultured semipelagic forms, one might suspect that differential larval growth is an adaptation to a benthic existence. The four species reported on here do not have pelagic nauplii, nor do they "drop" their eggs prior to hatching as reported for *Tigriopus californicus* by Huizinga (1971) and known to occur in some calanoid copepods (Zillioux and Gonzalez, 1972). Eggs mature in the egg sacs and red naupliar eyes are readily observed in the attached eggs just prior to hatching. Each nauplius hatches directly from the attached egg sac. Close observation of several specimens of *Paronychocamptus* during the hatching period revealed that the mothers actively prod and push their eggs releasing the nauplii one by one into the surrounding water. All nauplii gently settle to the substrate where some immediately start to feed and others become supine, firmly attaching themselves (ventral side down) to the substrate and are insensitive to careful probing. Most likely this dormant "settling" acts to prevent immediate growth and thus would account for the delay in naupliar development.

We do not know the causes of the delayed development nor can we test our hypothesis in the field since one needs to follow a particular individual's development. Whether the phenomenon is environmentally or genetically controlled is unknown. However, if the delayed development we have encountered in the laboratory also occurs in the field and it is genetically controlled, then those species that can differentially delay their larval development would have a great selective advantage over those species that cannot. If a species is able to "spread out" its developmental time in response to some condition, or delay its development in response to such a condition, it allows this species to: 1) spend a greater amount of time in that particular locale, extending its ability to utilize the resources and ensure a more equitable (noncrowding) population density; and 2) essentially modify its population growth-form from that of an *r*-strategist to that of a *K*-strategist. Harpacticoid copepods have traditionally been classified as a *r*-strategists *sensu* MacArthur & Wilson (1967), Pianka (1970) and Gill (1974), *i.e.*, large families and short life cycles (Heip, 1974). Indeed, the four species described here do have large families (for harpacticoid copepods) and would be expected to be *r*-strategists. However, upon hatching and between N_1 and N_3, the ability to develop slowly and have nauplii "appear" (*i.e.*, start development a few at a time) places only a few adults in the environment at any one time and most definitely favors efficiency of conversion of food with little or no waste (*i.e.*, *K*-strategy). If the population were to be environmentally controlled (*e.g.*, food-limited) and if the nauplii all developed at once and fed on the same resource at the same time, a real possibility exists that the resource would soon become limiting. By spreading out naupliar development and therefore not over-grazing the food resource (bacteria, diatoms) on sediment particles, the food resource would essentially be kept in the log-growth phase providing sufficient food for continued (and prolonged) naupliar development.

Smol and Heip (1974) suggest that a prolongation of the naupliar stages could be selective advantage in avoiding predation. They state that nauplii are less likely captured than copepodites by the predatory polyp, *Protohydra leuckarti*. If prolongation of the naupliar stages does not increase the length of the copepodite stages, then staying in the naupliar stages longer decreases predatory effects. We do not have *Protohydra leuckarti* at our sampling sites and therefore cannot specifically suggest it as the predator. However, Proseriata turbellarians are extremely common at our sampling sites (sometimes reaching 160–200 10 cm^{-2}) and are known to be voracious predators on harpacticoid copepods (Reinhard M. Rieger, University of North Carolina, personal communication). If the proseriats (or perhaps some other as yet unknown predator) are functioning similarly to *Protohydra* at our sampling sites, delayed naupliar development is one way of minimizing predation on these four harpacticoid copepods.

We do not know if the differential naupliar growth is genetically or environmentally controlled. The implications of such a naupliar development are, without doubt, extremely important to the success and propagation of the species. We are continuing the work and constructing life tables of the four species, to determine the exact stage when delay takes place and also to determine more acutely the factors controlling such a delay.

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SUMMARY

1. Four species of harpacticoid copepods maintained in culture appear to be able to inherently delay the duration of their naupliar stages.

2. Stage 1–3 nauplii continue to appear in culture up to 50 days after the mother hatches her eggs.

3. Those species with the delayed development essentially change from an r-strategist to a K-strategist during naupliar growth.

4. If delayed development also takes place in the field, then those species capable of delaying development in response to some "factor" have a distinct ecologic advantage over those that cannot.

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A HISTOCHEMICAL STUDY OF CEMENT SECRETION DURING THE INTERMOLT CYCLE IN BARNACLES

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Synthesis and accumulation of cement secretion in adult barnacles have been identified in specific regions in cement gland cells of several species (Lacombe, 1968; 1970; Lacombe and Liguori, 1969; Walker, 1970; 1974; Cheung and Nigrelli, 1972). Whether the cement secretion of the gland cells is a continuous or discontinuous process correlated with the physiological condition of the animal, however, has not been reported. Recently criteria for the determination of the molt stages of adult barnacles were developed (Davis, Fyhn and Fyhn, 1973). The present paper is a histological and histochemical study of cement gland cells in two acorn barnacles during the intermolt cycle.

MATERIALS AND METHODS

Specimens of *Balanus amphitrite* Darwin and *B. eburneus* Gould were collected at the dock of Duke University Marine Laboratory, Beaufort, North Carolina. The animals measured 10 to 15 mm (*B. amphitrite*) and 15 to 25 mm (*B. eburneus*) in basal diameter. Animals were fixed immediately upon collection or after a period of 5 to 31 days in aquaria. Animals were kept at 23 ± 1° C in the aquaria at a seawater salinity of 30 ‰. The animals were fed newly hatched *Artemia* nauplii (Metaframe, San Francisco Bay Brand) every second day. The water was changed weekly and at this time shells of the barnacles were cleaned by light brushing. Prior to fixation the animals were molt staged according to the method of Davis, Fyhn and Fyhn (1973). The number of specimens, their molt stage and days kept in aquaria are shown in Table I. The body and opercular valves were removed from the shell after cutting the opercular membrane. The shell together with the mantle tissue, cement glands and ovarioles was fixed for two hours in Carnoy's fixative without chloroform. In addition, Bouin's fluid (48 hours), Rossman's fluid (24 hours) and Carnoy's fixative (2 hours) were used as fixatives. After washing in 100% ethanol (Carnoy, Rossman) or in 70% ethanol (Bouin) the mantle tissues were dissected out, embedded in Paraplast (Fisher Scientific), and serial sectioned at 5 or 8 μ. For general orientation the Mallory-Heidenhain Azan stain (Koneff, 1938) was used. For histochemical studies the methods listed in Table II were applied. Linear dimensions were measured by a *camera lucida* (magnification 813 ×).

RESULTS

The cement gland cells of *B. amphitrite* are located in the mantle tissue among the ovarioles. The cells are rounded or oval with a diameter up to 200 μ. Each gland cell is connected to the cement duct system by a canal (Figure 1). This canal

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TABLE I

Number of specimens studied on Balanus amphitrite *and* B. eburneus *in various stages of the intermolt cycle.*

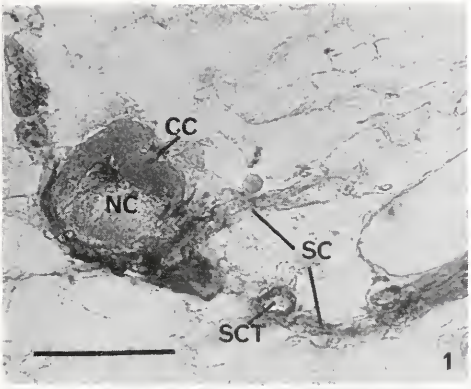
	Molt stages								Days in aquaria	Month of collection
	A	B_1	B_2	C	D_0	D_1	D_2	Not determined		
B. amphitrite	1	10		11		7	6		5–16	August
B. amphitrite								9	17–31	April
B. amphitrite		1	2		1		2	3	11–26	July
B. amphitrite		1	2	2		2	2		0	October
B. eburneus			1	2	2	1			0–2	August

corresponds to the secondary canal of the cement gland cells in *B. tintinnabulum* (Lacombe, 1968). From the joining point a collector canal runs peripherally in the cement gland cell in an infolding of its plasma membrane. The collector canal is branched or unbranched depending upon the size of the gland cell. The course of the collector canals was determined in serial sections of 25 cement gland cells. The cell size was given by the average of the largest and

TABLE II

Histochemical tests applied to the cement gland cells.

Compound tested for	Method	Control	Reference
General orientation	Mallory-Heidenhain Azan		Koneff (1938)
Proteins			
General proteins	Mercury-bromphenol blue		Pearse (1968)
Tyrosine containing proteins	Million reaction		Pearse (1968)
Protein bound SS-groups	Thioglycollate-ferric ferricyanide for SS		Adams (1956)
Protein bound SH-groups	DDD for SH	Alkylation	Barnett and Seligman (1952)
Protein bound SS- and SH-groups	Thioglycollate/DDD		Barnett and Seligman (1952)
Carbohydrates			
Acid mucopolysaccharides/Mucins	Toluidine blue 0	RNAse	Pearse (1968), Lillie (1929)
	Azur A	RNAse	Kramer and Windrum (1955)
	Mucihematein	RNAse	Laskey (1950)
	Alcian blue	RNAse	Steedman (1950)
1.2 glycol groups	Periodic acid Schiff's	Acetylation	Casselman (1962), Lillie (1965)
Nucleic acids			
DNA	Feulgen reaction/ Fast green		Feulgen and Rossenbeck (1924)
DNA and RNA	Methyl-green-pyronin Y	RNAse	Kurnick (1955)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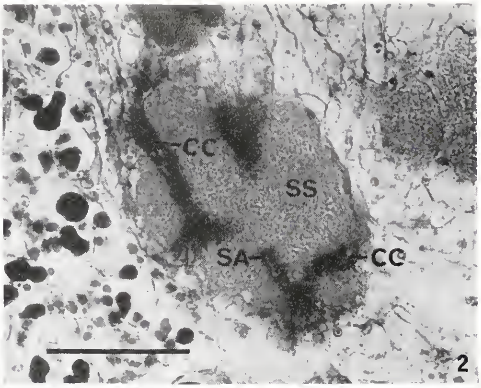

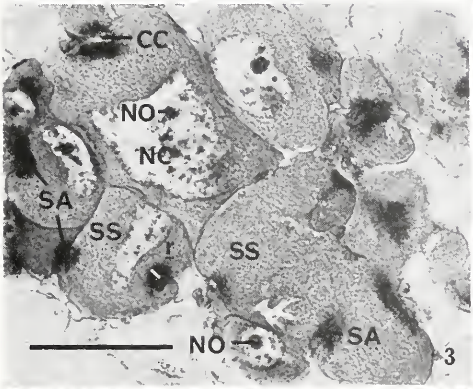
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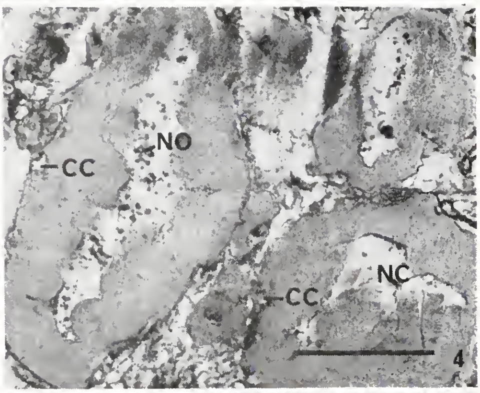

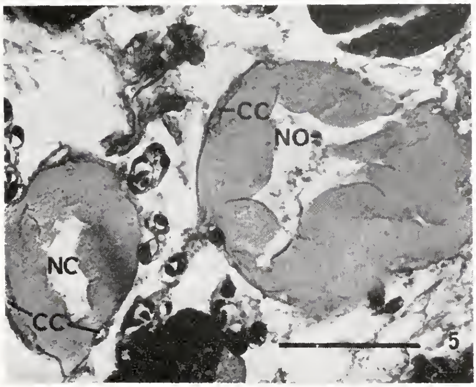

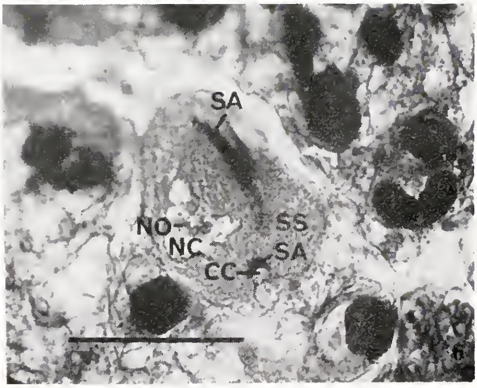
SA
NO
NC
SS
SA
CC
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FIGURE 1. Cement gland of *Balanus amphitrite*, stage A, fixed seven days after collection (Azan stains, section 8 μ). CC represents collector canal; NC, nucleus; SC, secondary canal; SCT, secondary canal transversely sectioned; marker is 50 μ.

FIGURES 2 to 6. Cement gland cells of *Balanus amphitrite* in various stages of the intermolt cycle, animals fixed immediately after collection (Azan stain, sections 8 μ). Figure 2 shows stage B_1; Figure 3 shows stage B_2; Figure 4 shows stage C; Figure 5 shows stage D_1; Figure 6 shows stage D_2. CC represents collector canal; NC, nucleus; NO, nucleolus; *r*, width of accumulation area; SA, secretion accumulation area; SS, secretion synthesis area; marker is 50 μ.

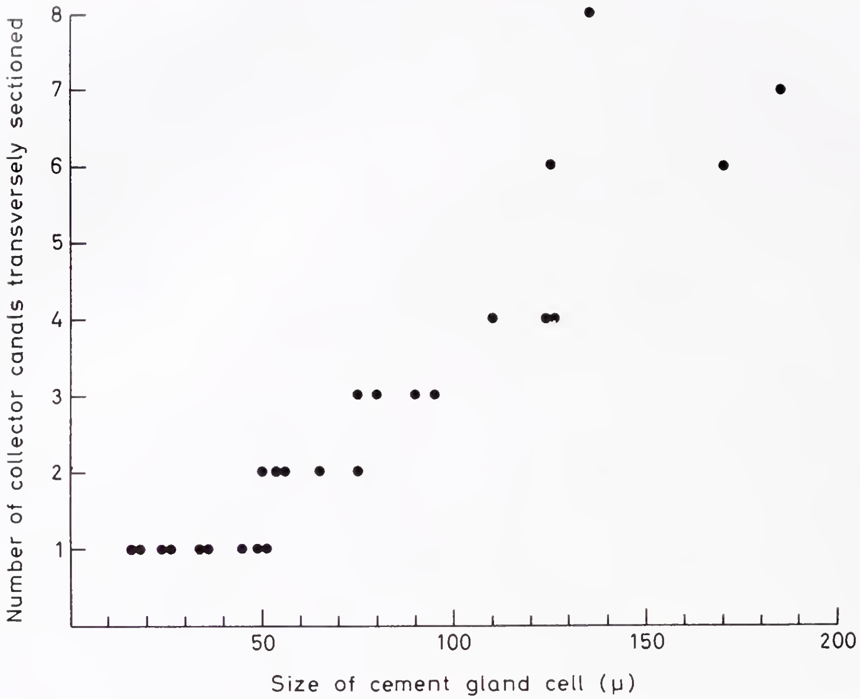
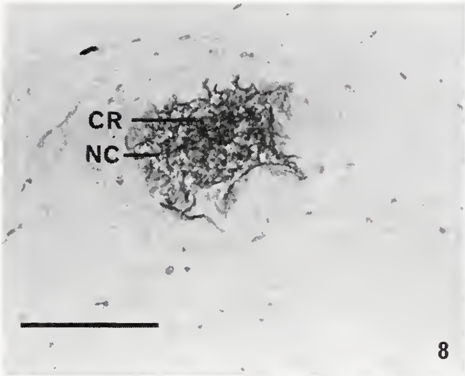


FIGURE 7. Number of collector canals transversely sectioned in cement gland cells of *Balanus amphitrite*. The size of the cells is given by the average of the largest and smallest diameter measured. Each point represents one gland cell.

smallest diameter. In gland cells up to 50 μ the collector canal is unbranched so that only one transversely sectioned canal may be observed. In cells of 50 to 75 μ the collector canal encircles the cell allowing the canal to be sectioned twice (Figure 3). In cells of 75 to 100 μ the collector canal appears to branch once so that three canals in transverse section were observed. In cells of 100 to 125 μ four transversely sectioned canals were observed. In cells of more than 125 μ the branching is more complex and the path of the collector canals is undulated which complicates the accurate determination of the number of canals. A minimum of six transversely sectioned canals were observed in serial sections of these cells. None of the collector canals seemed to have a "dead-end". The number of transversely sectioned collector canals in cement gland cells of various sizes are summarized in Figure 7.

The nucleus of cement gland cells up to 50 μ is rounded with a diameter of about 50% of the cell diameter (Figure 3). In larger cells the nucleus is irregular in outline making up 50 to 90% of the cell diameter in cross sections (Figures 3 and 10). The nuclei are rich in chromatin (Figure 8). In gland cells of less than 50 μ, one nucleolus was observed (Figure 3); in larger cells there are numerous nucleoli of various sizes (Figure 10). The cytoplasm appears dense and finely granulated without vacuoles. Sporadically, vacuoles were observed in the cytoplasm of gland cells fixed in Bouin's fluid. This is assumed to be a fixation artifact since Bouin's



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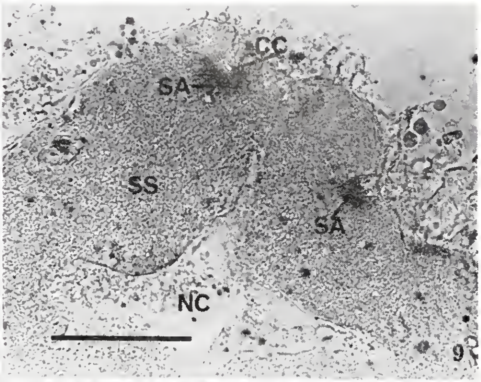

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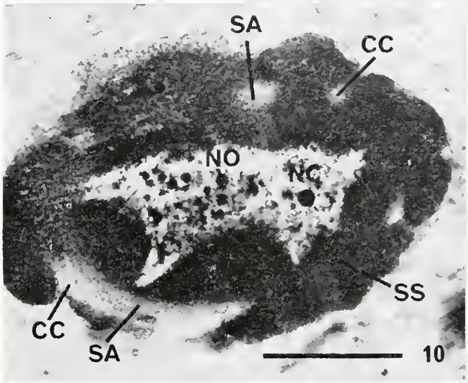

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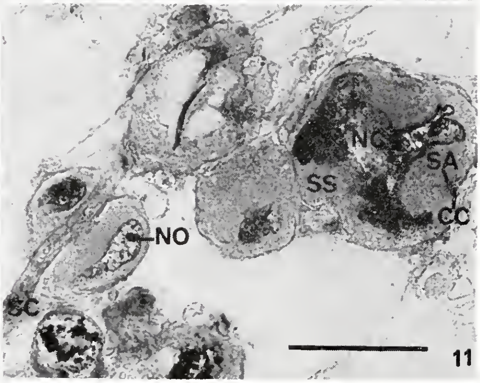

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FIGURES 8 to 10. Cement gland cells of *Balanus amphitrite* fixed immediately after collection (sections 8 μ). Figure 8 shows a cell in stage C stained by the Feulgen reaction; Figure 9 shows a cell in stage B_1 stained by Mercury-bromphenol blue; Figure 10 shows a cell in stage B_1 stained by Toluidine blue O. CC represents collector canal; CR, chromatin; NC, nucleus; NO, nucleolus; SA, secretion accumulation area; SS, secretion synthesis area; marker is 50 μ.

FIGURE 11. Cement gland cells of *Balanus eburneus*, stage B_2, fixed two days after collection (Azan stain, section 8 μ). CC represents collector canal; NC, nucleus; NO, nucleolus; SC, secondary canal; SA, secretion accumulation area; SS, secretion synthesis area; marker is 50 μ.

fluid has been reported to give vacuoles in a variety of tissues (Humason, 1972).

In animals fixed immediately after collection (Table I) the staining properties of the cytoplasm of the cement gland cells varied with the molt stage of the animal (Figures 2 to 6). In the postecdysial stage B_1 (Figure 2), two distinct regions within the cytoplasm were discerned by Azan staining: the region adjacent to the collector canals stained bright red while the remainder of the cytoplasm stained yellow to orange. These two regions correspond to the region of secretion accumulation and region of secretion synthesis, respectively, described for other barnacles (Lacombe, 1970; Walker, 1970). The size of the red areas was determined in cement gland cells of specimens of *B. amphtrite* in various stages of the intermolt cycle. The size was expressed by the width (marked by an *r* in Figure 3) of the red areas of transversely sectioned collector canals. The red areas do not change in width along the canal (Figure 2). No correlation was found between the

TABLE III

Width (μ) of secretion accumulation areas in cement gland cells of Balanus amphitrite *in different stages of the intermolt cycle. (The data are given as mean ± SE with the range in parenthesis of the 10 largest widths measured in 10 gland cells from each animal. Animals were fixed immediately after collection with Azan stain.)*

Animal number	Molt stage				
	B_1	B_2	C	D_1	D_2
1	11.3 ± 0.60 (9–15)	7.9 ± 0.28 (7–9)	0	0	3.7 ± 0.34 (2–5)
2	— (0–4)	1.2 ± 0.55	0	0.9 ± 0.02 (0.7–0.9)	2.3* ± 0.33 (2–3)

* Mean of three measurements.

width and the cell size. Table III shows the mean width of the ten largest areas measured in each animal. The size of the red areas was largest in stage B_1, decreasing during stage B_2, was zero in stage C and again increasing from D_1 to D_2. The areas staining red with Azan showed positive reactions in the protein tests (Table IV and Figure 9), but showed negative reactions for the carbohydrates tested (Table IV). The remainder of the cytoplasm showed negative reactions in the tests for proteins (Figure 9) and carbohydrates, and showed a strong positive reaction for RNA (Methyl-green-pyronin Y). This area stained dark blue by Toluidine blue O (Figure 10) and Azur A, and blue-green by Alcian blue (Table IV). After treatment with ribonuclease the cytoplasm was negative for Toluidine blue, Azur A and Alcian blue, indicating that the reactions in these tests were due

TABLE IV

Histochemical reactions of the cytoplasm of cement gland cells in Balanus amphitrite *showing secretion accumulation.*

Method	Secretion accumulation area	Secretion synthesis area
Azan	Red	Yellow-orange
Mercury-bromphenol blue	xxx	–
Millon reaction	x	–
Thioglycollate ferric ferricyanide	xx	–
DDD/Control	x/–	–/–
Thioglycollatic reduction, DDD	xx	–/–
Toluidine blue 0/Control	–/–	Dark blue/–
Azur A/Control	–/–	Dark blue/–
Mucihematein/Control	–/–	x/–
Alcian blue/Control	–/–	Blue-green/–
Periodic acid Schiff's/Control	–/–	–/–
Feulgen reaction	–	–
Methyl-green-pyronin Y/Control	–/–	xxx/–

xxx Strongly positive reaction
xx Moderately positive reaction
x Weakly positive reaction
– Negative reaction

to RNA. No true metachromasia was observed. In gland cells not showing red areas the cytoplasm adjacent to the collector canals was negative for proteins, RNA and carbohydrates. The remainder of the cytoplasm showed histochemical reactions similar to those found for the synthesis region in cells showing red areas (Table IV).

In *B. amphitrite* maintained in aquaria before fixation (Table I, Figure 1), no areas stained red by Azan were observed in cement gland cells irrespective of the size of the cement gland cell, the molt stage of the animal, or the number of days in aquaria (5 to 31 days). The gland cells showed the same histochemical reactions as cells lacking red areas in animals fixed immediately after collection. No difference was observed between gland cells of animals fixed in the different fixatives or in animals collected during spring and summer.

In *B. eburneus* the cement gland cells and the duct system had a histology similar to that found in *B. amphitrite* (Figure 11). The collector canals were running extracellularly in infoldings of the plasma membrane and the number of branches of collector canals increased with the cell size. Up to nine transversely sectioned canals were observed in the largest cells (diameter up to 180 μ). Small areas staining red by Azan were observed in cement gland cells of animals in stage B_2 (Figure 11) fixed two days after collection (width 2.9 ± 0.28 μ) and D_1 fixed after one day (width 4.4 ± 0.16 μ). No red areas were found in animals of stage C fixed after one day, and D_0 fixed immediately and after two days. The two regions of the cytoplasm showed histochemical reactions similar to those found for *B. amphitrite.*

DISCUSSION

The cytology of the cement gland cells in adult specimens of *B. amphitrite* and *B. eburneus* found in the present study is mostly in agreement with earlier descriptions of these species (Lacombe, 1970; Cheung and Nigrelli, 1972). The collector canals, however, were shown to be running extracellularly in infoldings of the plasma membrane and showed an increase in ramification with increasing size of the gland cell (Figure 7). Secretion accumulation could be found to an equally large degree along the collector canals in both species (Figure 2). More detailed investigations are necessary to determine whether the collector canals break up further into intracellular ducts as found in *B. balanoides* and *Elminius modestus* (Walker, 1970). The two regions in gland cells of *B. amphitrite* and *B. eburneus* showed histochemical reactions similar to those of the regions of secretion synthesis and secretion accumulation described for *B. eburneus* (Cheung and Nigrelli, 1972) and for *B. balanoides* and *E. modestus* (Walker, 1970).

Secretion accumulation in *B. amphitrite* seems to be correlated with the intermolt cycle. The accumulation could result from a synthetic activity starting in early proecdysis (D_1), increasing in D_2, reaching its maximum around ecdysis or in the postecdysial stage B_1, and decreasing to zero during B_2. The cyclic changes in the secretion accumulation could then reflect similar changes in the flow rate of secretion from the cement gland cells. In interecdysis (stage C) synthesis or accumulation seems absent. The amount of RNA in the synthetic region of cement gland cells was high in all molt stages, and no increase in protein concentration in this region could be detected in stage B_1. The applied techniques, however, do not adequately distinguish between small concentration differences. It seems unlikely that the accumulation should result from variations in flow rate of secretion from the cell with the secretion synthesis being constant, since not even traces of secretory product were observed adjacent to the collector canal in stage C animals (Figure 4).

In *B. eburneus* secretion accumulation was observed in animals in stage B_2 and D_1, but not in stage C and D_0. The areas were smaller than those appearing from earlier reports (Lacombe, 1970; Cheung and Nigrelli, 1972), but showed the same histochemical reactions as described for this species (Cheung and Nigrelli, 1972). The areas in *B. eburneus* in stage B_2 and D_1 were comparable in size to the areas in *B. amphitrite* in the same stages, and a molt cycle dependency of secretion accumulation is therefore possible also in this species.

The structural changes in the integument of adult *B. amphitrite* during the intermolt cycle (Davis, Fyhn and Fyhn, 1973) are similar to those described for malacostracans (Passano, 1960; Yamaoka and Scheer, 1970). The increasing amount of secretion in cement gland cells is thus coinciding with the deposition of the new exoskeleton. This raises interesting questions concerning the control of cement secretion in barnacles.

In specimens of *B. amphitrite* maintained in aquaria for 5 to 31 days before fixation, no secretion accumulation was observed in cement gland cells regardless of the molt stage of the animal. Animals in crowded populations of *B. balanoides* being lifted up from the substratum show cement droplets at their bases (Darwin, 1854). In *B. crenatus* and *B. glandula* cement was extended onto the base of animals separated from the substratum (Saroyan, Lindner and Dooley, 1970). Walker (1972) obtained droplets of cement from the base of *B. crenatus* and *B. hameri* kept with an airfilled space between the base and an underlying glass plate. In the present study no extended cement was observed on the bases of animals kept in aquaria. Newman, Zullo and Wainwright(1967), referring to unpublished data, reported *B. amphitrite* to reattach to glass slides. It is obscure why captivity apparently inhibited the secretory activity of the cement gland cells of *B. amphitrite* in the present study. However, it is possible that a minimum of contact between the animal and the substratum is required for cement secretion and extension to occur. A seasonal dependence of cement secretion can not be excluded since animals showing accumulation were collected in October, while animals not showing accumulation were collected during spring and summer. The present study shows that detectable secretion accumulation of cement gland cells in adult barnacles is not necessarily a species characteristic, but may be dependent upon exogenous as well as endogenous factors.

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SUMMARY

1. Regions of secretion accumulation and secretion synthesis were found in the cytoplasm of cement gland cells of adult *Balanus amphitrite* and *B. eburneus*. In contact with the accumulation regions collector canals were running extracellularly in infoldings of the plasma membrane and showed increasing ramification with increasing size of the cement gland cell.

2. In *B. amphitrite* the secretion accumulation was at its maximum in stage B_1 of the intermolt cycle, decreased during stage B_2, was zero in C and increased from D_1 to D_2. A similar variation in *B. eburneus* seemed probable.

3. The histochemistry of the accumulation and synthesis areas was studied.

4. In *B. amphitrite* maintained in aquaria before fixation, no secretion accumulation was observed in cement gland cells irrespective of molt stage of the animal.

5. The study shows that detectable secretion accumulation in cement gland cells of adult barnacles is not necessarily a species characteristic, but may be dependent upon endogenous as well as exogenous factors.

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OBSERVATIONS ON THE FOOD AND FEEDING BEHAVIOR OF ESTUARINE NEMERTEAN WORMS BELONGING TO THE ORDER HOPLONEMERTEA[1]

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The majority of nemerteans are carnivores or scavengers, but the exact nature of the food and the feeding habits of most species is poorly documented. Certain species belonging to two classes, Anopla and Enopla, capture and ingest whole animals from many of the invertebrate phyla (Hyman, 1951; Gibson, 1972). Under laboratory conditions, some nemerteans may be maintained on homogenized liver, starch paste, and cooked beef fat (Gibson, *op. cit.*). In the laboratory they also may be cannibalistic or may feed on other nemertean species.

Hoplonemerteans (Enopla), all of which have armed proboscides, in some cases strike a prey species with the proboscis and immobilize it with an injected toxin. As is the case in the larger heteronemerteans (Anopla), the prey is then either ingested whole (Roe, 1970) or, by other hoplonemerteans which feed on arthropods, sucked out of the exoskeleton (Hickman, 1963; Jennings and Gibson, 1969). Some hoplonemerteans are known to consume a variety of prey species, but some are such specialists that they may starve in the laboratory if not provided with the correct prey (Jennings and Gibson, 1969; Roe, 1970).

The purpose of this paper is to present laboratory observations on the capture and ingestion of live prey by three species of estuarine hoplonemerteans of the suborder Monostylifera. Their food, food preferences and feeding behavior will be compared with findings from similar studies on other monostyliferans, thus developing a more comprehensive understanding of feeding within the group, and defining more precisely the niches of these worms.

Materials and Methods

The three species studied were *Tetrastemma elegans* (Girard, 1852), *Zygonemertes virescens* (Verrill, 1879), and *Amphiporus ochraceus* (Verrill, 1873). They were identified according to the criteria described by McCaul (1963). All were collected during the summers of 1971, 1973 and 1974 from beds of eelgrass (*Zostera marina* L.) located at Gloucester Point, Virginia, where the salinity of the water was approximately 20‰. Marsh (1973) found both *Tetrastemma and Zygonemertes* in equal abundance in his study of the epifauna of eelgrass near Gloucester Point, but I found *Tetrastemma* to be far more common. *Amphiporus*, though not a rare species on eelgrass, is much less common than the other two (Marsh, 1973; Orth, 1971). Specimens of *Zygonemertes* were obtained also from among hydroids and bryozoans on wooden pilings at Wachapreague, Virginia, where the salinity was about 30‰. This species is the only one of the three that is found also on the Pacific coast of the United States (McCaul, 1963).

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[1] Contribution No. 724, from the Virginia Institute of Marine Science, Gloucester Point, Virginia.

Nemerteans living on the eelgrass were obtained by placing the freshly-collected grass into large rectangular or circular plastic containers and periodically removing the worms that surfaced around the periphery [see Kirsteuer (1967) for other details on collecting]. Once isolated, these worms were easily maintained for weeks in the laboratory without food or frequent water change.

The length of relaxed, moving specimens was determined by averaging several measurements taken with a millimeter scale. The latter was manipulated beneath a petri dish into which individual worms were placed.

Prey species used in these observations were crustaceans (amphipods, isopods and a mysid shrimp), polychaetes and molluscs. These were collected mostly from the eelgrass beds but some amphipods, especially members of the Corophiidae, were collected from scrapings of local pilings. Generally, only common species of prey were used in feeding preference tests. Amphipods, which were the most difficult of the prey to classify were identified with the aid of the descriptions and keys in McCain (1965), Feeley and Wass (1971) and Bousfield (1973). Total lengths of crustaceans (front of head to tip of telson) were measured with a calibrated ocular micrometer. Measurements of polychaetes were made to the nearest millimeter in the same manner used for the nemerteans.

Feeding tests were run with worms fresh from their habitat and also with those starved in the laboratory for known periods of time. Individuals of a prey species were presented to individual nemerteans in Syracuse watch glasses or in plastic compartmentalized covered boxes (length × width × depth: 51 × 43 × 39 mm, or 37 × 33 × 30 mm). Sometimes two different prey species were placed with each worm. Other procedures are described in the text. Water of approximately 20‰ salinity, usually filtered with a one micron filter, was used in the tests. Temperatures were 21–23° C. Tests were usually of short duration (*i.e.*, approximately 24 hours or less), but some were run for longer periods of time.

To observe the feeding behavior, predator and prey were placed into small dishes or depression slides. Use of a minimum amount of water promoted the desired contact between predator and prey. The complete sequence of events from the initial attack to the end of feeding was observed with a dissecting microscope. Individual events in the feeding process were timed with a stopwatch.

RESULTS

Tetrastemma elegans

This species fed only on amphipods, although presented with a wide range of possible prey (Table I). *Corophium acherusicum* was attacked and consumed much more readily than any of the others. As 90–100% of these crustaceans were usually consumed, the species became a useful control for most tests with other prey. Thus, the propensity to feed by an experimental population of *Tetrastemma* was easily determined by providing an aliquot sample with amphipods of this species.

The data in Table II show that *Tetrastemma* has a definite preference for *Corophium* over three other species of amphipods. In the first 15 minutes of Experiment 3, twelve individuals of *Corophium* were killed versus only two spec-

TABLE I

Summary of all feeding tests with Tetrastemma elegans, *involving one worm with one specimen of prey in each test.*

Species of prey	Number of prey		Per cent killed
	Presented	Killed	
Amphipoda			
Corophium acherusicum Costa	94	87	92.5
Ampithoe longimana Smith	19	2	15.5
Ampithoe valida Smith	2	1	
Cymadusa compta (Smith)	1	1	
Gammarus mucronatus Say	21	5	
Mixed (*Ampithoe* sp. and *Gammarus* sp.)	9	0	
Stenothoe gallensis (Walker)	6	0	
Caprella penantis Leach	36	6	16.7
Isopoda			
Erichsonella attenuata (Harger)	10	0	
Idotea baltica (Pallis)	17	0	
Edotea triloba (Say)	7	0	
Mysidacea			
Mysidopsis bigelowi Tattersall	7	0	
Polychaeta			
Nereis succinea (Frey and Leuckart)	9	0	
Sabella microphthalma Verrill	6	0	
Polydora ligni Webster	6	0	

imens of *Caprella.* There was a nine to one differential in Experiment 4, during the same time interval. Other numerous observations showed that if worms which were unresponsive to caprellids were presented with specimens of *Corophium,* they killed them at the first contact. An analysis of these data as well as all data dealing with *Corophium,* showed that there was no obvious selection by the worms for prey of a certain sex, or ovigerous females or smaller individuals.

Preferential feeding by *Tetrastemma* was tested further with other species of amphipods. Five specimens of *Corophium* (length: 3.13 mm mean, 2.28–4.05 mm range) were placed into each of five chambers, and one worm was added to each chamber. Two other chambers with five amphipods each but without worms served as a control for natural mortality. Predation was followed over a 20-hour period. Within five minutes all worms had attacked at least one amphipod; four had attacked more than one. At 15 minutes three had completed feeding and all of the other worms were feeding actively. At the end of the experiment, 19 of 25 amphipods were killed (4.56 corophiids/worm/day). Four other worms, each placed with five amphipods (mixed species: *Ampithoe valida, Gammarus mucronatus* and *Gammarus* sp.), killed a total of four individuals in 15 hours (1.6 amphipods/worm/day). Three of the four amphipods were *A. valida.*

The rate of predation on *C. acherusicum* by *Tetrastemma* was compared with that produced by *Zygonemertes virescens* on the same species. Two standard petri dishes (10 cm in diameter) were used to hold 25 amphipods each. Five worms of

TABLE II

Summary of experiments demonstrating food preferences of Tetrastemma elegans *for different species of amphipod prey.*

Experiment number	*Tetrastemma*		Amphipod prey			
	Number of worms	Mean length, mm	Species	Number	Mean length, mm	Number killed
1*	9**	9	*Corophium acherusicum*	9	—	8
			Ampithoe longimana	9	—	0
2*	9	8	*Corophium acherusicum*	9	—	9
	9	7	*Ampithoe* sp. and *Gammarus* sp.	9	—	0
3***	18	7	*Corophium acherusicum*	18	3.22	18
	18	7	*Caprella penantis*	18	5.15	5
4***	18	7	*Caprella penantis*	18	5.04	1
	18	7	*Corophium acherusicum*	18	3.07	13

* 24 hr experiment.

** One of each prey species with each worm.

*** 4 hr experiment; same worms used in experiments 3 and 4, but food presented to each reversed on successive days.

the first species were placed into one dish and five of the other into the second dish. All of these worms had been used in previous feeding experiments, and most had fed within four days prior to the beginning of the test; four specimens of *Tetrastemma* had fed the previous day. At the end of 18 hours, 19 of the 25 amphipods had been killed by *Tetrastemma* (5.05 amphipods/worm/day). Nine were killed within the first half hour. *Zygonemertes* killed twelve in 18 hours (3.20 amphipods/worm/day), but only two were killed in the first half hour.

The complete feeding process of *Tetrastemma* was observed many times in the laboratory. Most of the worms upon which the following account is based were 7 to 9 mm in length; the amphipods (*Corophium*) were 2.5 to 4.0 mm in length. Some observations were made also with other species of amphipods and larger worms.

Immediately upon contact of predator and prey, the armed proboscis is thrust out rapidly and usually strikes the amphipod somewhere on the ventral side. It may strike either in the anterior or posterior half of the prey. Occasionally the proboscis wraps around the body, but the tip still strikes ventrally. A single strike completely immobilizes the prey in from 15 to 90 sec (usually in less than 60 sec). The larger prey individuals are generally immobilized more slowly, but the size of the worm may be equally important with regard to immobilization time.

During this period of developing paralysis, the worm often moves away from the prey (up to a few worm-lengths) and then doubles back on its mucous-trail. It begins probing with its head, sometimes violently, on the ventral side of the amphipod, often everting the proboscis one or more times. The worm finally makes internal contact on the ventral side, probably penetrating between the sternal plates. Bleeding of the prey is occasionally discerned when an opening is produced. A large worm of 19 mm, feeding on *Gammarus mucronatus*, dislodged two thoracic gills in the process of probing and penetrating.

The head is inserted into the opening and the stomach is everted out of the rhynchodaeal pore, expanding to a hemispherical suctorial process. Feeding commences from 2.5 to 6 min (mean about 4 min) after the initial strike of the proboscis.

The body of the worm begins a series of peristaltic undulations while the ventral surface remains attached to the substratum. Muscular contraction of the body may be partially responsible for the suctorial action which begins to remove blood, tissue, organs, pigment, etc. from the prey. The flow of these materials can be seen through the transparent exoskeleton. Usually, all of the internal structures, including such organs as the eyes, are eventually sucked out leaving a completely transparent test. Occasionally the posterior part of the prey's digestive tract may remain intact, being the only opaque structure.

Feeding is completed in from 3.5 to 12 min with an average of about 7 min. The time taken for the whole process, *i.e.*, initial attack to the end of feeding, is approximately 6 to 17 min. The actual feeding time is greater for larger prey. Worms may stay with the prey for 1–2 min after feeding is discontinued, and then move away. Occasionally a worm may kill but not feed on the amphipod.

While completely sucking out the soft parts of ovigerous female corophiids, the worms never ingested the eggs or embryos. However, recently hatched young still living in the marsupium of the female were killed readily. Feeding on these minute animals (<1 mm long) is also suctorial, and takes place from the ventral side. Because they are so small, the worm's feeding time is very much shorter than the minimum figures noted above for adult amphipods.

To determine whether the immobilized prey is killed or only narcotized, starved worms (4 to 6 mm in length) were allowed to strike amphipods (*Corophium acherusicum*, 2.28–4.75 mm) with one thrust of the proboscis. The pair was separated and the amphipod was placed in clear sea water in a covered watch glass. Six pairs were treated in this manner. All of the six amphipods were immobilized in from 15–120 sec. None recovered during an observation period of several hours.

Zygonemertes virescens

A total of 20 worms was used for the following observations on *Zygonemertes*. Worms ranged in size from 12–40 mm (mean 23 mm). *Zygonemertes* attacked and fed on the amphipods and on one of the isopods (Table III). *Corophium* was attacked and killed more readily than the other species.

The following account of the feeding behavoir of *Zygonemertes* is based primarily on three worms consuming three amphipods (*Corophium*). The worms were 23, 25 and 30 mm long, while the amphipods were 3.7, 3.0 and 3.0 mm.

Upon contact with the prey, the proboscis was everted rapidly and penetrated the prey ventrally in two of three cases; in the other, it was a dorsal strike as the proboscis wrapped around the body of the crustacean. The amphipods were completely immobilized in 10 to 60 sec; the largest worm paralyzed its prey in the shortest time. In the latter case, the proboscis was attached to the amphipod for no longer than 2 sec. This indicates that the toxin is injected at the strike and not pumped into the "wound" by the proboscis. During the period of immobilization the worms moved away from the prey but kept contact by their posterior ends or by the attached mucus. Upon return to the prey, the worms actively probed the

TABLE III

Summary of all feeding tests with Zygonemertes virescens, *involving one worm with one specimen of prey in each test.*

Species of prey	Number of prey		Per cent killed
	Presented	Killed	
Amphipoda			
Corophium acherusicum Costa	41	29	70.6
Ampithoe longimana Smith	13	3	13.8
Gammarus mucronatus Say*	3	0	
Melita nitida Smith	13	1	
Caprella penantis Leach	17	6	35.2
Isopoda			
Erichsonella attenuata (Harger)	4	2	50.0
Idotea baltica (Pallis)	2	0	
Edotea triloba (Say)	3	0	
Polychaeta			
Nereis succinea (Frey and Leuckart)	14	0	
Gastropoda			
Bittium varium Pfieffer	3	0	
Crepidula convexa Say	3	0	

* This species is known to serve as prey for *Zygonemertes* (unpublished observations of T. A. Bernard, Jr., a former student).

ventral sides of the amphipods, exerted a few thrusts of the proboscis, penetrated, everted the stomach, and commenced sucking out all of the soft parts, leaving a transparent exoskeleton. The peristaltic action of the body was not as vigorous as in *Tetrastemma*.

In the case of the largest nemertean, the entire process from attack to end of feeding took less than 3 min. In the other two cases the whole process took 8.75 min and 9.75 min, actual feeding time being approximately 5 min in each case. The largest nemertean, which had entered the amphipod postero-ventrally, exited out of the anterior end. Amphipod embryos were not ingested. These observations further demonstrated the worms' preference for *Corophium*. In each case the worms had previously been kept in depression slides with *Ampithoe longimana* for 16 hours but had not fed. When specimens of *Corophium* were introduced, they were immediately attacked in all three cases.

Feeding on the isopod *Erichsonella* was also observed. The two isopods that were consumed measured 6.3 and 7.5 mm, and the worms were about the average length. These crustaceans were attacked with the proboscis, and appeared to be at least partially paralyzed almost immediately. The worms probed ventrally with their heads, eventually penetrating the exoskeleton. One worm penetrated at the base of the second left peraeopod, while the other worm inserted its head under the uropods in among the pleopods. In each case the head was inserted a short distance and the stomach was everted to produce a funnel-like suctorial organ. In one case the worm fed for 30 min, and then left after evacuating the anterior two-fifths of the isopod's body; the gut remained intact. The other worm was forcibly removed from the isopod after it had sucked out a part of the posterior end of the body.

Amphiporus ochraceus

Only seven worms were available for study, six collected from eelgrass at Gloucester Point and one at Wachapreague, Virginia on a wooden piling. The prey species tested were *Corophium acherusicum, Ampithoe longimana, Gammarus mucronatus*, and *Idotea baltica*. Six worms were starved for nine days prior to a 24 hour test. Each of three worms were given one *Ampithoe* and each of three given one *Gammarus*. One of each prey was killed and consumed. Another worm, starved for three days and then given two specimens of *Corophium*, consumed both animals within 24 hours. The embryos of the prey were not harmed. Three days later this worm consumed two more of the same amphipods within two hours. It failed to attack two specimens of *Idotea* within a two day period.

Observations on the feeding behavior of *A. ochraceus* are very limited in comparison to the other two species. It feeds by paralyzing its prey with thrusts of the proboscis, and in all cases fed from the ventral side of the amphipod. In one observation, a specimen of *Ampithoe* (5.7 mm long) was completely immobilized in less than 60 sec, and was then fed upon by suctorial action for five minutes. There was a slight peristalsis of the body, but it was less pronounced than in *Tetrastemma*. All amphipods were completely evacuated.

Discussion

Feeding behavior in the three species of nemerteans is very similar. It appears that the prey is found fortuitously. At least there is no general evidence that chemoreception or visual senses are involved, but critical experiments are needed to evaluate such stimuli. Contact must be made before the lethal trust of the nemertean's proboscis. Roe (1970) found that *Paranemertes peregrina* had to come in contact with a nereid worm or with its fresh products, such as a trail of mucus, before it would initiate the sequence of events in the capture and feeding process. Jennings and Gibson (1969) came to the same conclusion in dealing with the predator-prey relationship between *Amphiporus lactifloreus* and *Gammarus locusta*.

In all cases, the proboscis is everted rapidly onto the prey, sometimes wrapping around the body, and the stylet-bearing portion usually makes contact with the ventral side. One strike of the proboscis is ordinarily sufficient not only to paralyze but also to kill the prey. It appears that the venom of *P. peregrina* only paralyzes the polychaete prey (Roe, 1970). Presumably the stylet-bearing part of the proboscis in some way penetrates the integument of the crustacean and a toxin is transmitted into the hemocoel. It seems unlikely that the penetration of the exoskeleton is anything other than mechanical. Immobilization of the prey, as seen in the present study, is too rapid for any significant histolytic action to take place, although Jennings and Gibson (1969) suggested this mode of toxin entry in the case of amphipods attacked by *A. lactifloreus*. The proboscis is not used to draw the prey to the head of these hoplonemerteans as is the case with some of the heteronemerteans (Jennings and Gibson, 1969).

TABLE IV

Food of suctorial and macrophagous nemerteans belonging to the order Hoplonemertea, suborder Monostylifera.

Nemertean species	Prey species	Source
Suctorial feeders		
Amphiporidae		
Nipponnemertes pulcher (Johnston, 1837)	*Haploops tubicola* Liljeborg small polychaetes	Brunberg (1964); Berg (1972) Brunberg (1964)
Amphiporus lactifloreus (Johnston, 1928)	*Haploops tubicola* Liljeborg	Brunberg (1964)
	Gammarus locusta	Jennings and Gibson (1969)
Amphiporus ochraceus (Verrill, 1873)	*Ampithoe longimana* Smith *Corophium acherusicum* Costa *Gammarus mucronatus* Say	McDermott (this paper)
Zygonemertes virescens (Verrill, 1879)	*Ampithoe longimana* Smith *Ampithoe valida* Smith *Corophium acherusicum* Costa *Gammarus mucronatus* Say *Melita nitida* Smith *Caprella penantis* Leach *Erichsonella attenuata* (Harger)	McDermott (this paper)
Prosorhochmidae		
Geonemertes australiensis Dendy, 1892	young myriapods and small insects, particularly *Collembola*	Hickman (1963)
Tetrastemmatidae		
Tetrastemma elegans (Girard, 1852)	*Ampithoe longimana* Smith *Ampithoe valida* Smith *Cymadusa compta* (Smith) *Corophium acherusicum* Costa *Corophium simile* Shoemaker *Gammarus mucronatus* Say *Caprella penantis* Leach	McDermott (this paper)
Macrophagous feeders		
Emplectonematidae		
Paranemertes peregrina Coe, 1901	*Platynereis bicanaliculata* *Nereis vexillosa* *Neanthes brandti* *Lepidonotus squammatus* *Armandia brevis* *Syllis* sp. *Polydora* sp.	Roe (1970)
Ototyphlonemertidae		
Ototyphlonemertes brevis Correa, 1948	crustaceans and many setae of polychaetes in gut; fresh fish used as bait	Correa (1948)
Tetrastemmatidae		
Prostoma rubrum (Leidy, 1850)	oligochaetes (naids and others); insects, crustaceans and unicellular organisms; cannibalistic	Coe (1943)
	Aeolosoma sp. *Stylaria* sp. *Tubifex* sp. *Chironomus* larva	Jennings and Gibson (1969)
Prostoma lacustris	*Chironomus* and *Tanypus* larvae; *Cyclops*.	DuPlessis (1893) *from* Gibson, 1972

The relationship between size of prey, size of nemertean and lethal doses of toxin have not been determined quantitatively. Nevertheless, my observations reveal that the time for immobilization of the prey species is inversely related to the size of the nemertean and directly related to the size of the prey. These relationships could be quantified by accurately measuring the volume and dry weights of predator and prey.

The head of each nemertean species probes the ventral side of the paralyzed (dead) prey and eventually penetrates in this region. Here again penetration seems to be a mechanical process with the head wedging itself between the sternal plates. While it is possible that the probing head is continually secreting some lytic substance that softens the cuticular membranes, the rapidity of the penetration obviates such speculation.

It appears to be the stomach that is extruded through the rhynchodaeal pore, in the form of a bulbous sucker-like extension of the head. Peristaltic waves of the body are the external manifestation of a process producing the suctorial action which evacuates the whole exoskeleton. Although the stomach of *A. lactifloreus* is applied to various organs of the amphipod, the stomach of the present species usually remains in one position and there is a flow of organs and tissues into this organ. Again, the process is so rapid and continuous that histolytic secretions from the glands of the stomach wall participating in this phenomenon need not be postulated. Jennings and Gibson (1969) suggested that such secretions were utilized by *A. lactifloreus.* Overall, the feeding process described above is really very similar to that described in *A. lactifloreus* by the last authors. They also showed that *A. lactifloreus* will ingest dead specimens of *Gammarus* but without initial proboscis eversion. I did not attempt a confirmation of this behavior.

Other suctorial, arthropod feeders are the marine species, *Nipponnemertes pulcher* and the terrestrial form, *Geonemertes australiensis* (Table IV). Berg (1972) noted that after the former species kills the amphipod prey the stylet makes a hole between the ventral plates. He postulated that proteolytic enzymes are injected; the proboscis is then withdrawn, and the esophagus is everted into the hole and used to suck out the soft parts. Berg neither confirmed nor commented on Brunberg's (1964) observation that "small polychaetes" are also eaten by *N. pulcher.* If polychaetes are a regular part of its diet, this would be the only exception to the generalization that the suctorial species feed exclusively on arthropods.

When the insect prey of *Geonemertes* comes into contact with the viscous mucus surrounding the body of the worm, the proboscis is everted rapidly. The prey either adheres to the adhesive papillae of the proboscis or is wrapped within the proboscis. It is pierced eventually by the stylet of the proboscis and immobilized. Hickman observed that the rhynchostome is applied to the insect and the soft parts are sucked out, leaving the exoskeleton and any internal hard parts. Note that this worm differs from those discussed above in that the head of the worm is not inserted into the body and the anterior part of the intestinal tract is not everted.

Jennings and Gibson (1969) observed that ingestion of *Gammarus locusta* takes 30–40 min, whereas the whole feeding process in the three worms under present study was never longer than about 15 min. However, the size relationships of predator and prey need to be evaluated in order to document differences in feeding times.

The three hoplonemerteans studied here are all crustacean feeders, and with one exception, all feed primarily on amphipods. Indeed, one amphipod, *Corophium,* is attacked and ingested more readily than all others. At first I thought that the apparent preference for *Corophium* might be due partially to its relative sluggishness. *Caprella* also is quite sluggish, and it also appears to be more susceptible to predation than the laterally-flattened amphipods which move much more rapidly. However, many observations have shown that while numerous contacts with other amphipods may not induce a specimen of *Tetrastemma elegans* to attack, one or two contacts with *Corophium* will elicit an attack and capture by the same worm.

Jennings and Gibson (1969) showed that the freshwater species *Prostoma rubrum* was primarily an oligochaete feeder, paralyzing the prey and ingesting it whole. The two crustaceans they tested, *Gammarus* sp. and *Asellus* sp., were not harmed by *Prostoma.* Coe (1943) also lists oligochaetes as its preferred prey. This nemertean is, therefore, primarily an annelid feeder, but may also feed on slender, worm-like insect larvae such as the chironomids (Table IV).

The information in Table IV compares the present observations on the food of the Monostylifera with most of the findings in the literature. Practically all of these data were obtained from observations and experiments run in the laboratory with prey species from the same general habitat as the worms. The nemerteans are divided into suctorial and macrophagous feeders, but all paralyze or kill their prey with the stylet-bearing proboscis before they feed.

In the macrophagous nemerteans, annelids or worm-like aquatic insect larvae make up the known prey species. Oligochaetes are taken in fresh water (*Prostoma rubrum*) and polychaetes in the marine environment (*Paranemertes peregrina*). Only in the macrophagous species is it possible to identify the prey of freshly collected worms. The hard, secreted portions of the bodies of annelids or insects (*e.g.*, setae, jaws, denticles and exoskeletons) are readily seen and identified in the intestinal tract of compressed or dissected worms. On the other hand, at present it is not possible to identify the natural food in the intestine of the suctorial species. One therefore must carry out careful laboratory experiments with potential prey species in order to determine whether they actually are eaten.

In terms of the two feeding categories of Table IV, all members of the Amphiporidae for which information is available are suctorial. Members of the Tetrastemmatidae fall into each group, *i.e., Tetrastemma elegans* is suctorial and *Prostoma* is macrophagous. I prefer to withhold judgment on *T. melanocephalum* (Johnston, 1837), a species studied by Jennings and Gibson (1969). Only three worms were used for all of their observations, and these refused to attack or eat living annelids, crustaceans or molluscs. Jennings and Gibson noted similarities in the proboscis structure, stylet apparatus and gut structure of *T. melanocephalum* and *Amphiporus lactifloreus*, which suggest that the feeding process may be quite similar in the two species. Hence I suspect that when *T. melanocephalum* is studied carefully it may fall into the suctorial category with *T. elegans*.

Sanders, Goudsmit, Mills and Hampson (1962) examined the intestines of nine specimens of *Amphiporus* sp. (see Table 4, p. 68). In view of the evidence presented here that members of the genus *Amphiporus* are suctorial feeders of crustaceans, it is not surprising that they found seven with "empty" digestive tracts and two with only sand and benthic diatoms in their guts.

Roe (1970) noted that nereid worms show a distinct swimming escape response upon contact with the macrophagous species, *Paranemertes peregrina*. This response was not elicited upon contact with *Amphiporus formidabilis*, *Zygonemertes virescens* or *Emplectonema gracile*, nemerteans found in the same habitats as *Paranemertes* and the nereids. To her knowledge these three species did not prey on nereids. Information in the present study would confirm that *Zygonemertes* and probably *A. formidabilis* are crustacean feeders. According to Gibson (1972, p. 194) the genus *Emplectonema* is in the same family as *Paranemertes*. Further study is needed to determine whether it is also of the same feeding type.

The present observations make clear the necessity of laboratory study as well as field study if one is to delineate the feeding niches of suctorial nemerteans. For example, Marsh (1973) in his extensive study of the epifauna of the eelgrass (*Zostera marina*) community in the York River of Virginia, made erroneous inferences about the food of the suctorial species discussed in this paper. He identified a number of monostyliferans in this community with *Zygonemertes virescens* and *Tetrastemma elegans* being equally common. He suggested, referring to Barnes (1968), that they feed on polychaetes and other small invertebrates in the eelgrass, and in his diagram (Fig. 5, p. 95) of presumed trophic relationships, indicated that *Zygonemertes* feeds on the polychaete *Polydora ligni*. He indicated this prey presumably because it was the most common polychaete on the eelgrass. The evidence presented here shows that both of these nemerteans are in a different niche of the presumed food web, *i.e.,* they are primarily crustacean feeders with a decided preference for amphipods.

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SUMMARY

1. It is shown, by laboratory observations, that the marine hoplonemerteans *Tetrastemma elegans* (Girard, 1852), *Zygonemertes virescens* (Verrill, 1879), and *Amphiporus ochraceus* (Verrill, 1873), feed in a suctorial manner on small crustaceans.

2. Amphipods are the preferred crustacean prey, with an overwhelming preference being shown for *Corophium acherusicum* Costa, over several other species tested. *Tetrastemma* kills this amphipod at a greater rate than does *Zygonemertes.*

3. The prey is killed with a violent thrust of the proboscis, which in some manner punctures the exoskeleton and injects a toxin. The head is inserted into an opening produced on the ventral side, and the stomach is everted. A peristaltic action of the whole body is associated with a suction that removes nearly all of the internal contents of the prey.

4. The feeding behavior of the suctorial monostyliferans is compared with the macrophagous species. In at least one family, the Tetrastemmatidae, both feeding mechanisms are represented.

5. Laboratory studies on the food and food preferences of the suctorial species are essential in determining their particular ecological niches.

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LIPID COMPONENTS AND *IN VITRO* MINERALIZATION OF SOME INVERTEBRATE CARTILAGES

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Light and electron microscopic studies (Schaffer, 1930; Godman and Porter, 1960) as well as chemical investigations (see reviews by Irving and Wuthier, 1968, and Wuthier, 1973) have established the existence of significant lipid components in cartilages of vertebrate origin. Preliminary light and electron microscopic and chemical data have also indicated the presence of significant lipid components in invertebrate cartilage tissues (Person and Philpott, 1969a, b). Insofar as we are aware, however, there are no detailed analytical chemical data in the literature for invertebrate cartilage tissue lipid components. The present investigation was therefore undertaken as a beginning effort to provide such data.

In the course of the above investigation, as will be seen, it was found that the odontophore cartilage of *Busycon canaliculatum* was characterized by a high content of phosphatidyl serine. This observation was of great interest because it is now known that phosphatidyl serine plays an important role in both *in vivo* and *in vitro* mineralization of vertebrate cartilages (Irving and Wuthier, 1968; Wuthier, 1973). While invertebrate cartilages do not mineralize *in vivo* (Schaffer, 1930; Person and Philpott, 1969a), we have recently established by chemical and X-ray diffraction studies that *Limulus* gill cartilage can be induced to mineralize *in vitro* by incubation in a solution metastable for hydroxyapatite (Eilberg, Person and Zuckerberg, 1975). We have also found in unpublished experiments that other invertebrate cartilages will mineralize *in vitro* in the same hydroxyapatite-metastable medium (*i.e., Loligo* cranial or head cartilage and *Busycon* odontophore cartilage). The present study was therefore extended to include preliminary observations designed to detect possible relationships between the *in vitro* mineralizability of invertebrate cartilages and their phosphatidyl serine content.

MATERIALS AND METHODS

Tissue preparation for lipid analyses

Living specimens of the horse-shoe crab *Limulus polyphemus*, the marine snail, *Busycon canaliculatum*, and the squid *Loligo pealii* were obtained from the Supply Department of the Marine Biological Laboratory, Woods Hole, Massachusetts, in July, 1972. Also at this time, specimens of the "feather-duster" worm *Eudistylia*

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polymorpha were obtained from the Pacific Biomarine Supply Company, Venice, California, 90921. Animals were maintained in good physiological condition in running sea water tanks for 2–3 days prior to sacrifice. The following cartilage tissues were dissected and carefully trimmed of adherent tissues: gill cartilage from *Limulus* (8–10 inches body length); odontophore cartilage from *Busycon* (4–5 inches in shell length); cranial cartilage from *Loligo* (5–6 inches in mantle length); basal and tentacle cartilage of the crown of *Eudistylia* (4–6 inches in body length). Immediately following dissection and trimming, tissues were blotted with filter paper and wet weights were obtained. The tissues were then quickly cut into small pieces (3–4 mm on an edge) with scissors and sealed under nitrogen at atmospheric pressure, in glass vials containing a 2:1 (v/v) cold mixture of chloroform-methanol.

Analytical procedures

Fuller details and references for the analytical procedures outlined below are given in Rabinowitz, Luddy, Barford, Herb, Orlean, and Cohen (1967) and Rabinowitz, Bailey and Marsh (1971).

Lipids. All lipids or solutions containing lipids were kept under nitrogen at all times. Also, when stored, lipids were kept under nitrogen at −20° C in the presence of approximately 5 μg of butylated hydroxytoluene (BHT) per mg of lipid. Lipid standards were purchased from Applied Sciences, State College, Pennsylvania, and were tested for purity by duplicate chromatography, as described below. In no case did the level of impurities exceed 1%, and, in most instances, no detectable impurities were present. The tissue specimens were chopped finely and homogenized, following which they were extracted with chloroform-methanol (2:1, v/v). Only one extraction was required. Repeated extractions did not yield additional lipid material. One aliquot of the extract was subjected to silicic acid (Bio-Sil A, 100–200 mesh) column chromatography to separate neutral lipids (by chloroform elution) from phospholipids (by methanol elution). Individual lipid families were obtained from the separated neutral lipids and phospholipids by chromatography on thin layer plates and each was individually identified and assayed. Silica gel-G plates with petroleum ether-ethyl ether-acetic acid (90:10:1, v/v/v) were used for the non-polar lipids. At both sides of the spot with the unknown, known amounts of various authentic standards were placed. After developing, the area corresponding to each standard was scraped from the plate. Silica gel-H with chloroform-methanol-acetic acid-water (200:120–25:15, v/v/v/v) was used for the polar lipids. Each of the neutral and phospholipid families were hydrolyzed with alkali, and the isolated fatty acids converted to methyl esters, which were assayed by gas-liquid chromatography. The column used was 10% diethylene glycol succinate polyester, in a $\frac{1}{8}$ in., 6 ft. column, maintained at 20 lb. pressure of argon. Temperature programming was at 1° C per minute starting at 140° C until 190° C was reached.

DNA, RNA and protein. After extraction of lipids, the residue was isolated by centrifugation and washed with 5 ml of cold 5% trichloroacetic acid (TCA). Five ml of 5% TCA were added to the remaining residue and the mixture heated at 90° C for 15 minutes. After cooling, the solution was filtered. The soluble material was transferred to a volumetric flask for use as the stock solution for the assays of collagen, DNA and RNA.

Non-collagen protein. The biuret technique was utilized for protein assays. These were done on 1 ml aliquots of the stock solution. Bovine serum albumin (Nutritional Biochemicals, Cleveland, Ohio) was used as the standard.

Collagen. Collagen was assayed by a micro-biuret method. "Tendon-collagen" (Sigma Chemical Company, St. Louis, Missouri) was used as the standard. Samples were hydrolyzed and hydroxyproline assayed by chromatography.

DNA and RNA. DNA was assayed by the diphenylamine reaction while RNA was determined using the orcinol technique. "Purified DNA and RNA" (Nutritional Biochemicals) were used as standards.

Mineralization experiments

Gill cartilages from *Limulus*, cranial cartilages from *Loligo* and odontophore cartilages from *Busycon* were dissected from live animals and trimmed of adherent extraneous tissues. Slices were cut by hand in dimensions of 0.5–3.0 mm thickness, and placed in a solution metastable for hydroxyapatite (Eilberg, Gould and Sobel, 1965), prepared as follows: to a one liter volumetric flask was added 100 ml of a solution containing $NaHCO_3$ (220 mM/l), KCl (50 mM/l) and NaCl (702 mM/l). Next, 50 ml of a solution containing Na_2HPO_4 (25.1 mM/l) and NaH_2PO_4 (6.45 mM/l as $NaH_2PO_4 \cdot H_2O$) was added. Following this, about 200 ml of distilled water was added. CO_2 was then bubbled through the solution for one minute. Next, 11 ml of a $CaCl_2$ solution (250 mM/l as $CaCl_2 \cdot 2H_2O$) was added, and the flask made to mark with distilled water. N_2 was bubbled through the resulting solution until a pH of 7.30 was reached. (Attempts to induce mineralization of invertebrate cartilages in calcium carbonate-metastable media have been unsuccessful thus far). Tissue incubations were carried out at 37° C for 10, 16, 24, 32, 48 and 96 hours, using 50 ml Erlenmeyer flasks, filled to the brim with the above medium, and closed with stopcock grease-sealed rubber stoppers. Control incubations were carried out in the same media at 20° C, at which temperature it has been established that mineralization does not occur (Eilberg *et al.*, 1975). At the end of the incubation period tissues were removed from the medium and some were quickly rinsed in distilled water, gently blotted, and allowed to air dry. Others were placed in 10% buffered formalin, processed through paraffin embedding, cut without demineralization at 5–6 μm thickness, and stained with 0.1% aqueous toluidine blue or by the Von Kossa silver technique. Air-dried specimens were used to prepare Debye-Scherrer X-ray diffraction patterns, by Dr. A. Hirschman, Department of Anatomy, Downstate Medical Center, Brooklyn, New York.

RESULTS

Chemical analyses

Table I summarizes over-all chemical composition of the invertebrate cartilages, whose variability undoubtedly reflects a) the pleomorphic nature of the respective tissues, especially in respect of their relative cell and matrix content as revealed by histologic analysis (Schaffer, 1930; Person and Philpott, 1969a), and b) differences in physiologic and growth status of the different organisms at the time of sacrifice. The various tissue collagen contents appear to correlate well with the relative amounts of cells and matrix in the respective tissues, as disclosed by histo-

TABLE I

Chemical components of invertebrate cartilage tissues
(wet weight basis; average of 3 analyses ± SEM).

	Limulus (gill)	*Eudistylia* (crown & tentacle)	*Loligo* (cranial)	*Busycon* (odontophore)
% RNA	0.60 ± 0.10	1.31 ± 0.09	0.36 ± 0.07	0.09 ± 0.01
% RNA	0.09 ± 0.03	0.16 ± 0.03	0.09 ± 0.01	0.06 ± 0.02
% Collagen	4.32 ± 1.46	19.05 ± 2.17	6.35 ± 0.43	1.01 ± 0.23
% Non-collagenous protein	19.52 ± 3.74	18.73 ± 2.45	10.12 ± 1.94	3.68 ± 1.85
% Lipid	0.11 ± 0.08	0.39 ± 0.17	0.22 ± 0.12	0.80 ± 0.34

logic studies reported by the above authors. Also, while all the tissues contain hydroxyproline, none of the respective collagens have as yet been purified, so that analyses of purified samples are not yet available. The comparisons between tissues and with the vertebrate tendon collagen standard, are therefore not rigorously quantitative and are only suitable for rough comparison purposes. With the above in mind then, *Eudistylia* crown cartilage has most collagen (19%), while *Busycon* odontophore cartilage has least collagen (1%). The collagen contents of the cartilages from *Loligo* (6%) and *Limulus* (4%) are intermediate between the above values. It should also be mentioned that in the case of *Limulus* and *Loligo,* at least, there are varying amounts of chitin (not quantitated) in the cartilages of these animals, as established by our (unpublished) confirmation of earlier observations by Lankester (1884) and Halliburton (1885).

Tables II and III summarize data on lipid composition of the invertebrate cartilage tissues. It can be noted that in contrast with difficulties experienced in lipid extraction of vertebrate cartilages (Irving and Wuthier, 1968), in the present

TABLE II

Lipid components of invertebrate cartilages.

	Limulus (gill)	*Eudistylia* (crown & tentacle)	*Loligo* (cranial)	*Busycon* (odontophore)
1. *Lipids*				
Neutral lipids (% of total)	70.00 ± 12.84	65.31 ± 12.93	64.35 ± 12.35	26.91 ± 12.31
Phospholipids (% of total)	30.00 ± 5.91	34.69 ± 4.43	35.65 ± 4.54	60.00 ± 4.99 13.09 (unknown associated with phospholipid fraction)
2. *Neutral lipid components*				
% Mono- & diglycerides	3.41 ± 1.14	3.30 ± 1.37	2.38 ± 0.71	1.28 ± 0.36
% Triglycerides	15.89 ± 3.17	10.84 ± 2.81	7.42 ± 1.34	0.71 ± 0.21
% Free fatty acids	9.39 ± 1.49	11.88 ± 9.74	30.13 ± 12.39	6.90 ± 1.45
% Methylated fatty acids	10.09 ± 3.74	6.16 ± 0.94	2.73 ± 1.03	1.95 ± 1.21
% Cholesterol (or steroid-like)	2.89 ± 0.83	11.44 ± 6.31	2.71 ± 0.91	11.96 ± 2.49
% Esters of steroid-like substances	28.56 ± 12.99	21.69 ± 12.49	18.98 ± 2.77	4.11 ± 3.98
3. *Phospholipid components*				
% Phosphatidyl choline	10.00 ± 7.85	3.55 ± 0.99	8.73 ± 1.43	15.11 ± 2.76
% Phosphatidyl serine	1.23 ± 1.01	1.64 ± 0.81	2.00 ± 1.00	13.74 ± 5.84
% Phosphatidyl ethanolamine	0.34 ± 0.12	5.19 ± 2.31	5.40 ± 2.35	10.23 ± 3.97
% Lysolecthicin	0.01 ± 0.01	0.82 ± 0.43	0.78 ± 0.43	1.36 ± 0.49
% Phosphatidic acid	12.32 ± 5.54	20.76 ± 3.94	16.34 ± 7.44	14.73 ± 2.11
% Sphingomyelin	0.89 ± 0.63	1.37 ± 1.10	0.95 ± 0.39	3.71 ± 1.79
4. *Unknown neutral lipids and phospholipids*	4.98	1.37	1.45	14.21

TABLE III

Fatty acid composition of invertebrate cartilage lipids.

Per cent fatty acid	*Limulus* gill cartilage		*Eudistylia* crown cartilage	
	Pooled phospholipids	Pooled neutral lipids	Pooled phospholipids	Pooled neutral lipids
8:0	—	—	—	—
10:0	—	—	0.56	—
12:0	—	—	0.74	2.49
12:1	3.50	2.81	—	9.98
14:0	trace	5.79	3.32	3.99
14:1	5.84	0.92	0.46	4.49
16:0	50.75	37.05	30.19	44.00
16:1	6.08	1.50	4.92	1.99
16:2	trace	trace	0.22	trace
18:0	23.83	28.91	27.47	5.99
18:1	—	trace	13.56	3.99
18:2	—	trace	2.31	—
18:3	trace	—	2.92	—
20:0	—	—	0.68	—
20:1	—	9.36	2.21	1.25
20:2	—	—	1.38	—
20:3	—	—	trace	—
22:0	—	—	0.68	3.49
22:1	—	trace	1.00	trace
22:2	—	—	0.46	—
24:0	—	—	—	—
Unknown	10.00	13.66	6.92	18.35

study, as mentioned in the section on materials and methods, no difficulties were encountered with the lipid extractions of the invertebrate cartilage tissues. This suggests that in the invertebrate tissues, lipids are probably not covalently bound, as in some noncartilaginous vertebrate connective tissues such as those of skin (Rabinowitz and Shapiro, 1973) but exist rather in loose physical associations with other tissue components. Indeed, the lipid patterns shown in Tables II and III resemble patterns of lipid composition reported for noncartilaginous vertebrate connective tissues (Rabinowitz, Luddy, Barford, Herb, Orlean and Cohen, 1967; Rabinowitz, Rutberg, Cohen and Marsh, 1973; Rabinowitz and Hercker, 1974). It is therefore interesting that the invertebrate cartilages do show significant differences in comparison with vertebrate cartilage tissues. Thus, invertebrate tissue levels (Table II) of lecithin, triglycerides and phosphatidyl ethanolamine are much lower, and levels of phosphatidic acid much higher than in vertebrate cartilages (Irving and Wuthier, 1968; Wuthier, 1973). Also noteworthy in this context is the unusually high value for the phosphatidyl serine component of phospholipids found in *Busycon* odontophore cartilage, 13.7%, as compared with values between 1.2–2.0% for the cartilages of *Limulus, Eudistylia* and *Loligo.*

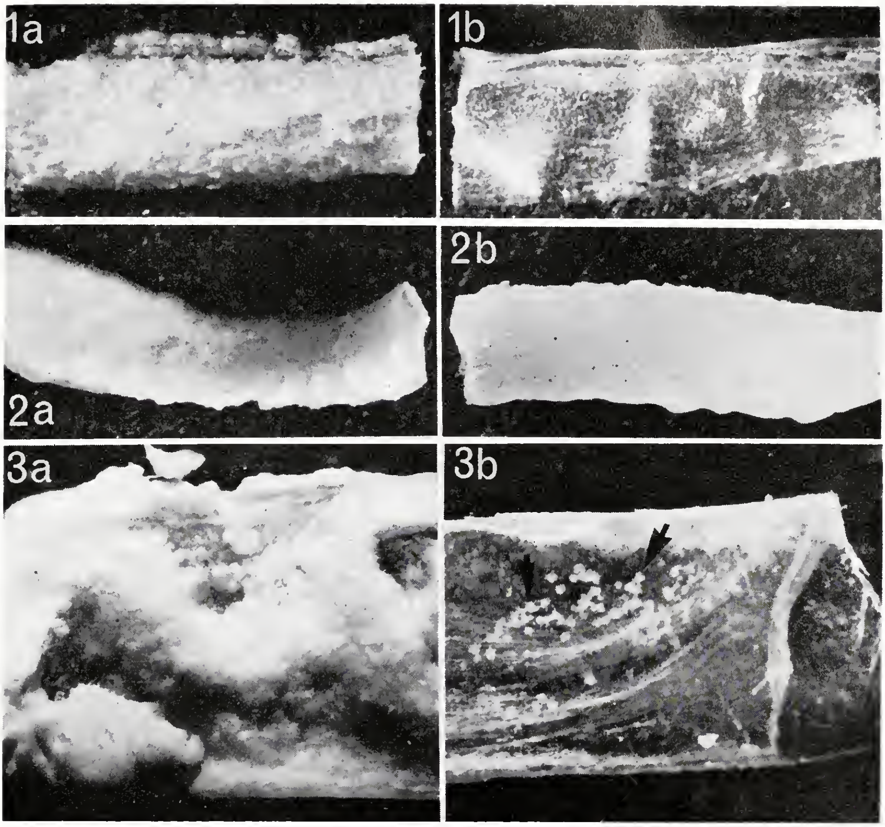
Individual fatty acid determinations were made for the lipids of *Limulus* and *Eudistylia* cartilages (Tables III). The more saturated fatty acids (palmitic, stearic, oleic) were found to be approximately two to three times more abundant than unsaturated fatty acids in these invertebrate cartilages. This is approximately the same ratio observed for lipids of vertebrate collagens (Nikkari and Heikinen, 1968) and is somewhat surprising because in most marine organisms (both vertebrate and invertebrate) unsaturated fatty acids are usually much higher than saturated ones (Markley, 1960). The presence of as yet unidentified lipid components in all tissues is of interest, and attempts will be made to identify these components in future experiments. It may also be noted that significant amounts of methylated fatty acids were found in all tissues. At times, the presence of methylated fatty acids may reflect artifacts of transmethylation from solvent methanol used in extraction procedures. However, Leikola, Nieminen and Salomaa (1965), Louh and Garton (1970), and Rabinowitz, Tavares and Marsh (1972) have shown that nonspurious, significant values for methylated fatty acids are found in a variety of tissues under conditions in which artifacts of transmethylation cannot occur. In the present experiments, the values for methylated fatty acids are believed to be valid because it has been established in earlier experiments that the techniques employed would not lead to transmethylation (Louh and Garton, 1970; Rabinowitz, Tavares and Marsh, 1972).

Mineralization experiments

The *in vitro* mineralization of invertebrate cartilage is visible to the unaided eye as a crystalline phase deposited within and upon the surface of the incubated tissues. The typical appearance of well-mineralized cartilages is illustrated in Figures 1, 2, and 3, which show respectively, air dried specimens of mineralized (a), and nonmineralized (b) controls from *Busycon, Limulus* and *Loligo*, after 96 hours of incubation.

After 10 and 16 hours of incubation, unaided eye examination of tissue specimens from each of the three organisms revealed that mineral deposits were apparently lacking in all. These observations were confirmed by histologic and X-ray diffraction examinations of the 10 and 16 hour-incubated tissues, which also could not detect evidences of mineralization.

After 24 hours of incubation, however, signs of mineralization were detectable by the unaided eye in *Busycon* odontophore cartilage, but not in *Limulus* or *Loligo* cartilages. Staining of alternate serial sections from the 24 hour incubated tissues by toluidine blue and by the Von Kossa technique, confirmed that there was mineralization of the *Busycon* cartilage, but no mineral deposits were seen in sections of either the *Limulus* or *Loligo* cartilages. Figure 4 is a photomicrograph of a toluidine blue-stained section of *Busycon* odontophore cartilage (24 hours incubation) showing the appearance of granular mineral deposits within cells (*mc*), matrix (*mm*) and also within the periochondrium (*mp*). Figures 5 and 6 show, respectively, comparable photomicrographs of toluidine blue-stained sections from *Limulus* and *Loligo* cartilages also after 24 hours incubation. No signs of mineralization are detectable in the latter two specimens. In black and white photomicrographs of toluidine blue stained sections, as shown in Figure 4, mineral appears as black granules. Von Kossa stain of alternate serial sections show positive reactions in these same granules. After 32 hours incubation, first signs of mineralization of both *Limulus* and *Loligo* cartilages were seen. At this time the mineralization of *Busycon* cartilage was much farther advanced than that of *Limulus* or *Loligo*.



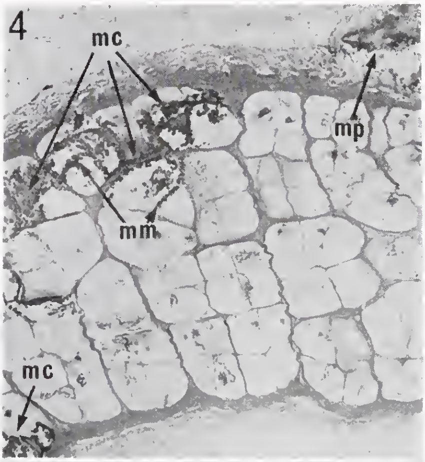
FIGURES 1–3. Air-dried specimens of invertebrate cartilages after 96 hours incubation, to illustrate appearance of (*a*) well mineralized and (*b*) non- or poorly-mineralized control tissues. The mineralized and control tissues in each figure were removed from the same animal, and incubated in the same media, except that temperature of incubation was 37° C for mineralized specimens and 20° C for controls. At the latter temperature, mineralization is strongly inhibited or does not occur at all.

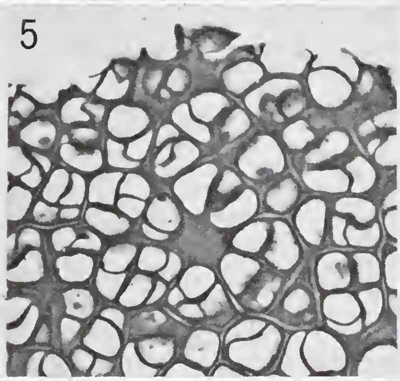
FIGURE 1. *a* (*left*) : *Busycon* odontophore cartilage, impregnated and incrusted with white, opaque, hydroxyapatite crystals; *b* (*right*) : translucent appearance of non-mineralized control specimen; seven × magnification.

FIGURE 2. *a* (*left*) : *Limulus* gill cartilage, impregnated and incrusted with white, opaque, hydroxyapatite crystals; *b* (*right*) : non-mineralized control cartilage. The latter tissue surface is smooth, and although the tissue also appears opaque, it is in fact more translucent than the mineralized tissue, but this translucency was not captured by the camera; seven × magnification.

FIGURE 3. *a* (*left*) : *Loligo* cranial cartilage, impregnated and incrusted with white, opaque crystals of hydroxyapatite; *b* (*right*) : control specimen, non-mineralized except for a small number of crystal aggregates (arrows); nine × magnification.

After 48 hours incubation, mineralization had progressed in cartilage specimens from all three animals, and was most advanced, still, in *Busycon* tissue, as judged by relative amounts and distribution of mineral. After 96 hours of incubation, mineralization in each of the three tissue types was so widespread, that it was difficult to discern significant differences between them in respect of quantity and dis-





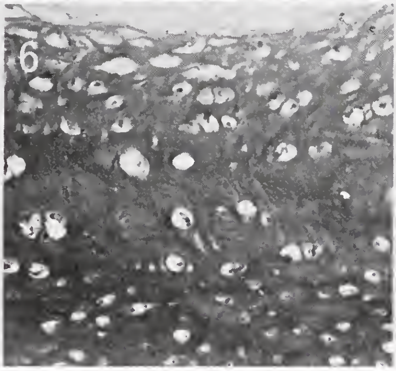


FIGURE 4. Photomicrograph of *Busycon* odontophore cartilage section (24 hours incubation) showing distribution of mineral granules within cells (*mc*), in matrix (*mm*) and in perichondrium (*mp*); toluidine blue stain, 153 × magnification.

FIGURE 5. Photomicrograph of *Limulus* gill cartilage section (24 hours incubation) showing lack of mineral deposition in the tissue; toluidine blue stain, 153 × magnification.

FIGURE 6. Photomicrograph of *Loligo* cranial cartilage section (24 hours incubation) showing lack of mineral deposition in the tissue; toluidine blue stain, 153 × magnification.

tribution of mineral. Detailed studies of chondrocyte and matrix alterations during mineralization, as a function of incubation time, are now in progress and will be reported in a separate paper. X-ray diffractions made from air-dried, well-mineralized (*i.e.*, 96 hour incubation) cartilage specimens from each of the three animals used in this study, gave in each instance a typical pattern of a poorly crystallized biological hydroxyapatite, as illustrated in a preceeding publication (Eilberg, Person and Zuckerberg, 1975, figure B).

DISCUSSION

It is not possible at this time to interpret the variability of lipid and other chemical components reported for the different cartilage tissues, from an evolutionary or phylogenetic standpoint. Only after a larger data base has been accumulated can such an attempt be made. At present, only the differences in collagen content of the respective cartilages appear susceptible of some interpretation, based upon the relative matrix content of the tissues as revealed by histologic studies. Thus, *Eudistylia* cartilage containing most collagen (19%) has large sheets of matrix with relatively few cells (Person and Mathews, 1967). *Loligo* cartilage, next in respect of collagen content (6%) is a typical hyaline cartilage (Philpott and Person, 1970). *Limulus* and *Busycon* cartilages containing 4% and 1% collagen respectively are of the "chondroid" variety of cartilage, and possess decreasing amounts of matrix (Shaffer, 1930; Person and Philpott, 1969b; Person and Philpott, 1967).

Of special interest was the demonstration that while invertebrate cartilages do not mineralize *in vivo* in the course of their natural history, they possess the capability to be induced to mineralize *in vitro*. Two features of this *in vitro* mineralization are worthy of mention here. First, until now only hydroxyapatite mineral phases can be formed in the invertebrate cartilage tissues and not calcium carbonate, which is the mineral of widest natural occurrence and distribution in invertebrate skeletal tissues. However, this may be the result of lack of a suitable metastable medium for calcium carbonate deposition, and the search for such a medium continues. Secondly, our data show that the *in vitro* mineralization by hydroxyapatite occurs earlier and with greater intensity in *Busycon* odontophore cartilage than in either *Limulus* gill or *Loligo* cranial cartilages. This is of interest because *Busycon* cartilage phospholipid content is 13.5% phosphatidyl serine, as compared to 1.2% and 2.0% phosphatidyl serine in *Limulus* and *Loligo* cartilages, respectively. This suggests a possible role for phosphatidyl serine in mineralizability of invertebrate cartilages, perhaps similar to its demonstrated role in mineralization of vertebrate cartilage (Irving and Wuthier, 1968, Wuthier, 1973). Studies designed to answer this question are now in progress.

Finally, it may be suggested that the ability to induce mineralization of invertebrate endoskeletal cartilage tissues creates a new experimental approach to a major problem of animal evolution, namely, the lack of mineralized endoskeletal cartilage structures in invertebrates, as compared with vertebrates. It may now be possible to deal with both genetic and environmental influences which may play roles in repression (or activation, if such can be achieved) of the ability of the invertebrate cartilage tissues to mineralize *in vivo*.

We thank Dr. Richard Libbin and Mr. Rober Ozer for their assistance, and Dr. Albert Hirschman for preparation of X-ray diffraction patterns.

SUMMARY

Cartilage from several invertebrate organisms *Limulus polyphemus* (Arthropoda), *Eudistylia polymorphia* (Annelida), *Busycon canaliculatum* (Mollusca) and *Loligo pealii* (Mollusca) were assayed for RNA, DNA, collagen, noncollagenous protein and lipid content. The variability in levels of collagen (1–19%) in the respective tissues, appeared to correlate well with their relative matrix content as revealed by previous histologic study. Determinations were made of mono- and diglycerides, triglycerides, free fatty acids, methyl esters, steroid-like materials and steroid-like esters, phosphatidyl choline, phosphatidyl serine, phosphatidyl ethanolamine, lysolecithin, phosphatidic acid, and sphingomyelin. The fatty acid composition of the major sub-groups was determined, and it was found that the ordinary fatty acids, palmitic, stearic, and oleic represented the largest fraction, whereas values for unsaturated fatty acids in the invertebrate cartilages were smaller than those usually reported for marine organisms. Significant levels of phosphatidyl serine were found in all tissues, being especially high in the odontophore cartilage of *Busycon, i.e.,* 13.7% of phospholipid content. Some unusual and as yet unidentified fatty acids, representing approximately 10% of the total fatty acid composition were also found.

In vitro mineralization of *Busycon* odontophore cartilage, *Limulus* gill cartilage and *Loligo* cranial cartilage were carried out by incubation of the respective tissues at 37° C in a medium metastable for hydroxyapatite. Of the three tissues mentioned above, earliest signs, and most rapid mineralization occurred in the *Busycon* cartilage, which also has the highest content of phosphatidyl serine. This latter observation is of interest because of the demonstrated role of phosphatidyl serine in mineralization of vertebrate cartilages, and suggests the possibility of a similar role in the *in vitro* invertebrate cartilage mineralization.

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LIFE HISTORY AND PREDATOR–PREY INTERACTIONS OF THE NEMERTEAN *PARANEMERTES PEREGRINA* COE

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This study concerns the life history of the predatory nemertean *Paranemertes peregrina* Coe, predator-prey relations of *P. peregrina,* testing of predator strategy models, and a consideration of the effects *P. peregrina* has on community structure. *P. peregrina* occurs from Ensenada, Mexico through the Aleutian and Commander Islands (Coe, 1940), and also in Japan (Yamaoka, 1940). It lives in both muddy bays and rocky intertidal areas, and eats polychaetes (Roe, 1970).

Nemerteans are common in intertidal areas of temperate regions. Most are carnivorous (Coe, 1943) and, because of their size and abundance, may be important organisms in marine communities. No complete investigation of the feeding ecology of a free-living nemertean has been done, and since the great majority of nemerteans are free-living, such a study should add greatly to the knowledge of the phylum.

Materials and Methods

Three study areas were chosen: two mud flats on San Juan Island, Washington, and the ballast along the ferry landing at Edmonds, Washington.

The study site at Snug Harbor, Mitchell Bay (48° 34′ 9.5″ N, 123° 9′ 48″ W), San Juan Island included the area between −0.2 m and −0.4 m, where *P. peregrina* was common. (A tidal height of 0 equals mean lower low water, as recorded in the U. S. Coast and Geodetic Survey Tide Tables.) The fauna contained mainly polychaetes, tanaids, and thalassinids, plus much ulvoid algal cover most of the year. Only the superficially dwelling polychaetes, with certain exceptions, were available as prey for *P. peregrina*, and included the nereid *Platynereis bicanaliculata,* the hesionid *Ophiodromus pugettensis,* the opheliid *Armandia brevis*, the spionid *Scolelepis fuliginosa,* syllids, polynoids, and phyllodocids. Polychaetes living in deep tubes included the nereid *Nereis vexillosa,* lumbrinerids, terebellids, maldanids, glycerids, nephtyids, ariciids, and dorvilleids.

The study area at Garrison Bay (48° 34′ 57″ N, 123° 9′ 17″ W), San Juan Island, Washington, extended from −0.1 to −0.5 m. Polychaetes and many clams comprised the major infauna and algae were much less common here. Polychaetes included the superficially dwelling *P. bicanaliculata, A. brevis, O. pugettensis,* syllids, at least five species of spionids, and phyllodocids; plus deeper dwelling *N. vexillosa,* lumbrinerids, terebellids, maldanids, glycerids, cirratulids, ariciids, sabellids, and oweniids. *Aglaja diomedea* (cephalaspid gastropod), common at both mud flats, attained high densities in summer and overwintered as juveniles just below the mud surface.

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The study area at Edmonds, Washington, (47° 48′ 12″ N, 122° 22′ 56″ W) consisted of boulders 1.5 to less than 0.3 m high, piled along the north face of a ferry landing. Tide level was from +2.1 m at the top of the highest rock to about +0.2 m at the top of the lowest. Boulders were covered with a thick coat of *Balanus cariosus* and *Mytilus edulis*. Syllids (*Typosyllis* sp.) were most common in clumps of larger barnacles, and mussels and spionids (*Boccardia* sp.) were most abundant in cracks between small barnacles. Other polychaetes included phyllodocids, lumbrinerids, the chrysopetalid *Paleanotus chrysolepis*, polynoids, and very few nereids.

In Washington lower low tides between September and January occur during hours of darkness; those from April through July occur during daylight hours. February, March and August have both daylight and nighttime lower low tides. Most field work was carried out during lower low tidal periods from April, 1968 through August, 1970.

Densities of active *P. peregrina* were determined by counting numbers/m^2 in randomly selected (or all) square meters along transects on the substratum surface during the time of maximum nemertean activity (within an hour after the tide recedes). Transects were chosen haphazardly with respect to nemertean densities. If few worms were active, all worms seen were counted and collected. Each month the numbers/m^2 of active nemerteans in each of five permanently marked 10×4 m^2 mud flat areas were counted to get density measures through time in given places.

In alternate months four $\frac{1}{8}$ m^2 mud samples from each mud flat were dug, the numbers of *P. peregrina* on the surface were counted, then all nemerteans in sieved mud were counted to determine total density. The per cent of active worms was determined by dividing the number on the mud surface by the total number. Since sieving mud was so time consuming, not enough samples were taken to get statistically significant results.

For size-frequency studies, the first 50–100 worms were collected from the density transects each month. In the laboratory their length and width were measured after allowing each worm to extend to its normal crawling length; their sex was determined by observing gametes through the ivory colored venter.

To determine what *P. peregrina* had been eating, another 50 worms (also along the density transects) were collected each month. After being brought to the laboratory, these worms were immediately isolated into small jars, half full, of clean sea water, and after 48 hours were measured, sexes were determined, and faeces were identified and preserved in 70% alcohol. Faeces of *P. peregrina* are easily identified bundles of polychaete setae and jaws in mucus.

Nemerteans used for measurement and faecal analysis were returned within a few days to approximately the places within the study areas from which they were collected. Areas were cleared of *P. peregrina* to study movement and total densities. To measure growth and movements, individuals dyed with neutral red (making the white ventral streak pink) were put into permanently marked areas. Every month the number of marked worms still in or near the given square meter were counted, collected and measured, and returned to the same m². The ventral streak remained pink for at least eight months.

Population densities of the major prey, *Platynereis bicanaliculata*, were determined during alternate months at Snug Harbor and Garrison Bay. Mud was collected into numbered jars (20 cm² surface area at opening) or plastic freezer containers (surface area 100 cm²) at regular intervals throughout the study area. In the laboratory, the contents of the containers were emptied with sea water into a pyrex dish and agitated until all nereids swam to the surface of the water. They were counted and collected, and nereids from the samples with the most nereids were preserved in 70% alcohol. The preserved nereids were later measured for size frequency analysis.

Other prey species' densities were determined as follows: specimens of *Armandia* and spionids were counted in most of the nereid samples. Very few spionids were seen in these samples, so in August, 1970, all the spionid tubes seen per 1/100 m² while diving at high tide at Snug Harbor were counted. *Nereis vexillosa* densities were estimated from the numbers collected in $\frac{1}{8}$ m² mud samples taken for total *P. peregrina* counts. At Edmonds, barnacles and mussels were scraped off small patches of rock, and in the laboratory the volumes of the clumps were determined, and they were broken apart and the polychaetes found were counted.

To determine activity periods of *P. peregrina* dives using S.C.U.B.A. equipment were made at each study area, day and night, and at all tides except lower low tide to see if, and how many, nemerteans were active other than when uncovered at lower low tides. The dispersion pattern of *P. peregrina* was determined by the method described by Pielou (1959), using the formula $\pi D \dot{w} = a$.

During several low tides the behavior of the animals was observed, especially with respect to foraging, feeding, time required to feed, and movements. For behavioral observations artificial mud flats were made in shallow concrete outdoor aquaria. The aquaria were about 1 m² in size, and each contained a wooden frame about 5 cm smaller per side than the aquarium. Water came through hoses above the aquaria, and a plug at the bottom of each aquarium outside the wooden frame could be pulled for "low tides." Because of the inner frame, water had to percolate through the mud while the mud flat was draining; it did not simply run off the surface of the mud. Nemerteans responded as if there was a real low tide when water was thus forced down through the mud on its way out. The activity cycles of individual nemerteans were observed in these small mud flats.

Two similar indoor mud flats were also made and kept going for ten months. One contained nereid food for the nemerteans but no "low tides" were made, to determine if *P. peregrina* would eat under water. The other contained no nereid food and was established to determine how long specimens of *P. peregrina* can withstand starvation. In both experiments, running sea water was used, and no data were collected to determine if nemerteans absorbed dissolved food from the water itself. Nemerteans from each flat were removed once each month, measured, weighed, and then returned. Jaws of nereids that were consumed in the natural habitat of *P. peregrina* were collected from the faeces and measured using a calibrated compound microscope.

Laboratory feeding experiments were done in two ways. First, adult nemerteans do not feed very well if simply left in a container with prey; prey must be placed directly in front of a nemertean's head before it is recognized as prey. Each day adults were presented with as many nereids as they would eat and results were recorded. Secondly, young nemerteans will eat if enough nereids are included in the container to be detected. For a year three juvnile nemerteans were grown in pint jars with nereids in a refrigerator kept near 7° C and the number of nereids eaten was recorded.

Diversity of prey species was calculated as $H' = -(n_i/N) \ln (n_i/N)$ (Pielou, 1966). Significant differences in H' were determined by the method of Pielou (1966).

Dietary selectivity was calculated by applying Ivlev's (1961) "electivity coefficient." This coefficient, E, is a ratio of the amount that a food type is eaten to its abundance or availability, $E = (r_i - p_i)/(r_i + p_i)$.

Observations and Results

Population life history of P. peregrina

Lengths of *P. peregrina* are correlated with dry weight ($r = 0.77$, $P < 0.001$), but length × width (area, mm^2) is more highly correlated with dry weight ($r = 0.93$, $P < 0.001$) and this index is the size unit used throughout. Linear regression of length × width on dry weight is: area = 2.11 dry weight + 50.33. Figure 1A and B gives sizes of *P. peregrina* through time at Garrison Bay. Roe (1971) shows sizes of individuals through time at Snug Harbor and Edmonds and sizes of Garrison Bay individuals during other months.

Gamete production. The presence of developing eggs and sperm can be determined 3–4 months before the gametes are ripe. In all three populations the total number of females significantly outnumbered the total number of males: Snug Harbor, 452 females, 196 males, $\chi^2 = 101.1$, $P < 0.005$; Garrison Bay, 105 females, 52 males, $\chi^2 = 17.9$, $P < 0.005$; Edmonds, 147 females, 29 males, $\chi^2 = 79.1$, $P < 0.005$. Even during the peak spawning seasons females outnumber males about two to one (Fig. 2; see Roe, 1971 for more detail). At Snug Harbor and Garrison Bay part of the population consists of non-ripe animals at peak spawning periods also (Fig. 2; Roe, 1971).

Spawning. The major spawning season for all three populations is spring or summer, and minor spawing periods occur throughout fall and winter in the mud flat populations (Fig. 2 for Snug Harbor; Roe, 1971, for Garrison Bay and Edmonds populations). At Garrison Bay the major spawning period is in April, and a few worms spawn in November (Roe, 1971). At Snug Harbor most spawning occurs in June, with some also in November (Fig. 2); and at Edmonds spawning occurs in March and April only (Roe, 1971). The major spawning season for each population usually lasts about a month; spawning is not higly synchronous within a population; and the dispersion pattern of *P. peregrina* is random during spawning season.

Eggs and sperm are shed through temporary gonopores along the dorsolateral surfaces of adults. In the laboratory, spawning sometimes resulted in death of the adult. Adults kept after spawning lived for six weeks and then died from poor laboratory conditions.

Coe (1940, p. 286) said, "gelatinous clusters containing many ova (are) deposited during the warmer months of the year," by *P. peregrina*. Although spawn-

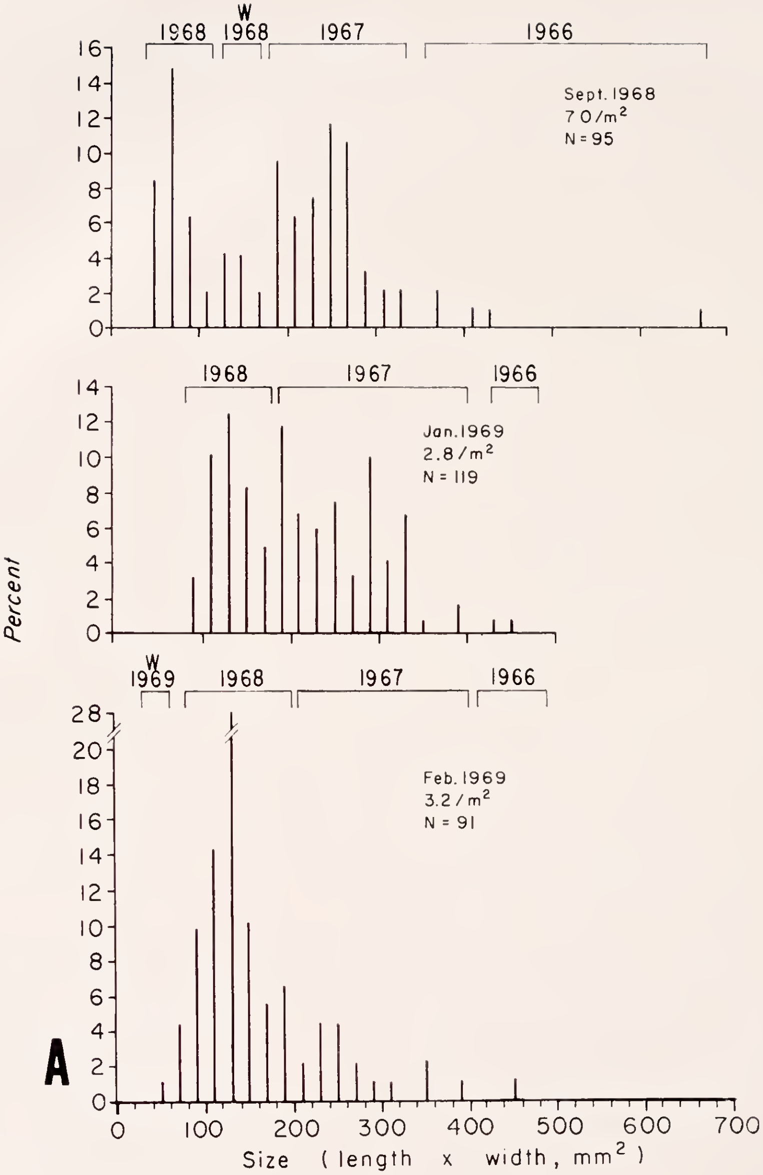
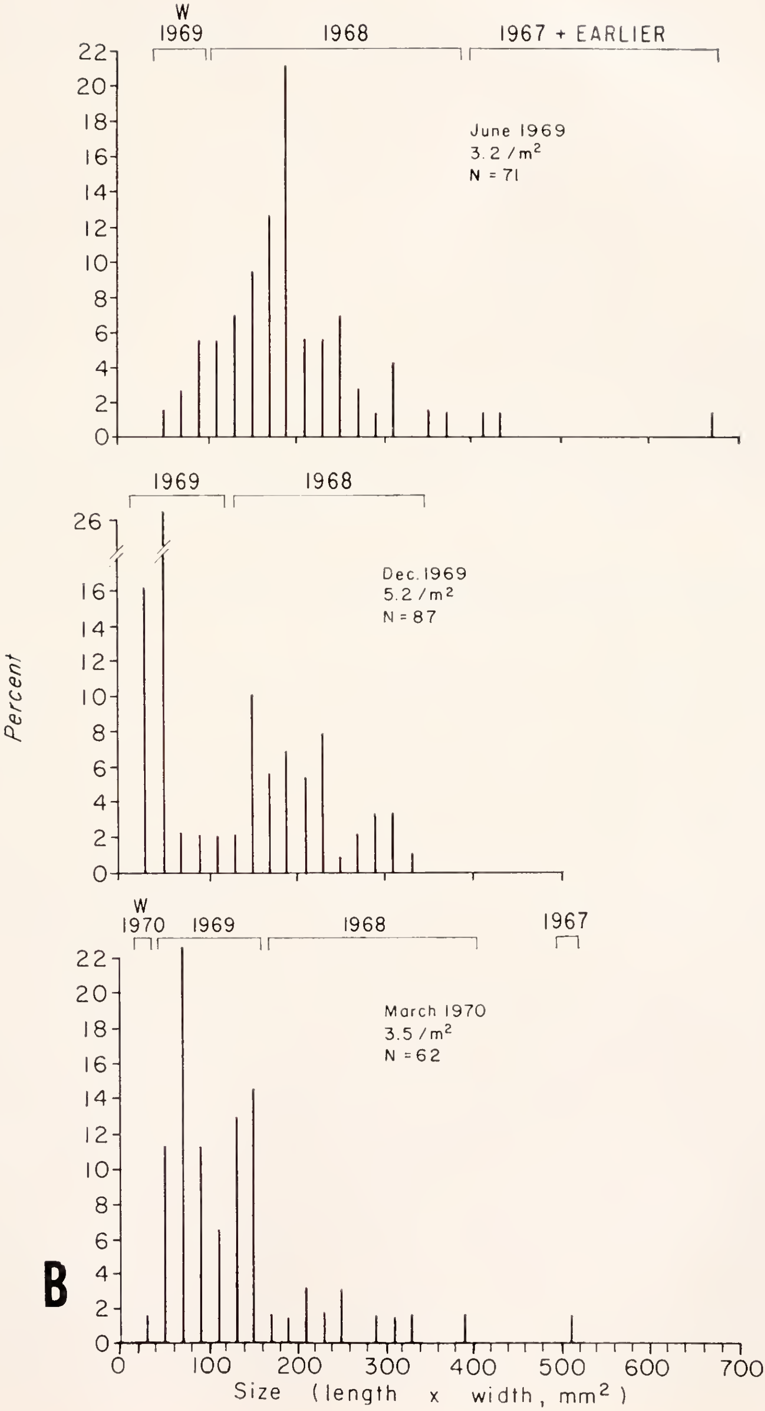


FIGURE 1. A) Size-frequency distribution of *Paranemertes peregrina* at Garrison Bay, September 1968, January 1969, and February 1969. Size class interval is 20 mm^2. Number/m^2 is the average total *P. peregrina* density. N is the number in the sample from which the size-frequency histogram was drawn. W equals winter recruits. B) Size-frequency distribution of *Paranemertes peregrina* at Garrison Bay is continued; June 1969, December 1969, and March 1970. Number/m^2 for June 1969 is the average *P. peregrina* density during low tide. The December 1969 sample includes an area of unusually high density of juveniles, thus increasing the percent in small size categories.


W
1969
1968
1967 + EARLIER
June 1969
3.2 /m^2
N = 71
1969
1968
Dec. 1969
5.2 /m^2
N = 87
W
1970
1969
1968
1967
March 1970
3.5 /m^2
N = 62
Percent
0
2
4
6
8
10
12
14
16
18
20
22
26
0
100
200
300
400
500
600
700
Size (length x width, mm^2)

B

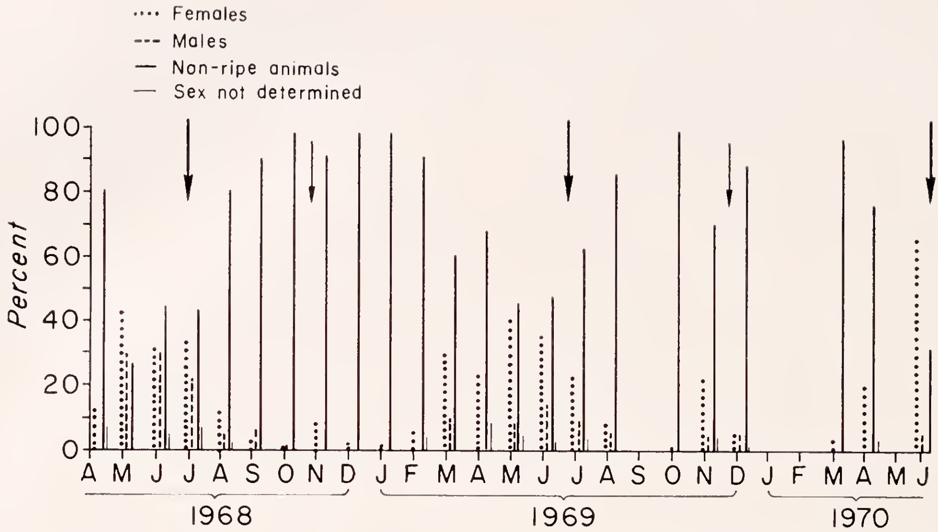


FIGURE 2. Per cent of the *Paranemertes peregrina* population at Snug Harbor comprised by males, females, and non-ripe animals each month. Large arrows indicate major spawning periods; small arrows indicate minor spawning periods.

ing was not observed in nature, the eggs of those that spawned in the laboratory did not form gelatinous masses. Extensive examination of all types of egg masses observed at both Snug Harbor and Garrison Bay showed none to be nemerteans. Single eggs of *P. peregrina* were also collected in the plankton of Mitchell and Garrison Bays in June, 1969. So, at least in the areas used for this study, *P. peregrina* spawns eggs and sperm into the sea.

Development. Eggs are yolky and about 235 μ in diameter; with the fertilization membrane and jelly coat, 650 μ. They are blastulae in 30 hours. Larvae start swimming within the jelly case after about two days and hatch the third day when kept near 10° C. In two weeks the accessory stylets and basis rudiment of the proboscis have formed. There is continuous change from a ciliated tear-drop shaped, weakly swimming larva with two anterior flagella to an elongate, muscular, benthic form. Gradually, the tiny worm spends more and more time on the bottom until finally it cannot swim any longer. Larvae can stay in the plankton for at least eight weeks, but develop faster if food is available. The cultured worms started feeding in the laboratory on 3-segmented specimens of *Platynereis bicanaliculata* (about 0.2 mm long) when they were about one month old and less than 1 mm in length. Spionid larvae were also offered as possible food, but juvenile specimens of *P. peregrina* were never observed or induced to eat one. Although larvae were not specifically examined for food, those younger than about four weeks were never seen with any food material in the developing gut. It is assumed that they live on yolk reserves until they start feeding on polychaetes. Young specimens of *Nereis vexillosa* quickly grew too large to be eaten by nemertean juveniles and showed the typical nereid swimming escape response to *P. peregrina* (Roe, 1970), even when the nemerteans were still in the flagellated swimming stage. Even as one-month old

TABLE I

Growth of three individuals of Paranemertes peregrina *in the laboratory.*

Date	Size		Number of animals measured
	Area (l × w, mm^2)	Length	
June 23, 1969	0.3 mm, egg diameter		3
August 11, 1969	—	0.15	1
August 21, 1969	—	0.4	1
October, 1969	<7, 6, <7	0.7, 1.1, 0.7	3
November, 1969	12.5, 13.5, 20	2.5, 2.7, 2.9	3
December 7, 1969	16.5–23.1, 36	3.3, 4, 3.3	3
December 17, 1969	41.6–46.8	5.2, 5.2	2
December 24, 1969	52, 54, 51	5.2, 5.4, 5.1	3
January 15, 1970	46.2, 52, 54	4.2, 5.2, 5.4	3
January 24, 1970	54.9, 58.2, 75	6.1, 6.5, 6.8	3
February 20, 1970	65–70	6.5–7	1*
February 27, 1970	112.5–120	7.5–8	1
March 8, 1970	120	8	1
March 22, 1970	*ca.* 135	9	1
April, 1970	*ca.* 180	12	1
May 24, 1970	285	15	1
June, 1970	310–350]	14–16	1**

* Largest of three young animals died February 8, 1970; it was already developing eggs when it died. One of the other two died in June, 1970; it quit feeding or growing in February, 1970, and became a ripe male.

** The third animal lived until December, 1970, at which time she spawned eggs and fragmented. In July, 1969, she pinched off part of her tail, so growth was not recorded thereafter.

worms less than 1 mm in length, *P. peregrina* has the strong food preference for nereids present in the adults; likewise, nereids show the escape response to *P. peregrina* throughout their lives. That the escape response is species-specific was reaffirmed when 3-segmented nereids showed aggressive rather than escape behavior toward a 2.5 mm specimen of *Zygonemertes virescens.*

Growth. In the laboratory three nemerteans were grown from eggs starting on June 23, 1969, until their deaths (Table I). They exhausted their food supply at the end of August and were not fed again until mid-October. Then they grew about 1 cm in length per month from October through March, 3 cm per month for the next two months, and then stopped. The sole survivor was 15 cm long at the end of a year (Table I). After the initial slow growth when they did not have food, the sizes of these laboratory-grown worms corresponded with the sizes of young in nature during the fall (Fig. 1A, September and 1B, December).

Adult nemerteans can also withstand periods of low food. The 14 nemerteans in the indoor artificial mud flat that contained no nereids generally lost weight and grew smaller each month, but one was still alive after nearly 7 months (October 1 to April 29). The average area decreased from 100 mm^2 for 14 worms in October to 58 mm^2 for 2 worms in December. Two worms collected in March averaged 74 mm^2; they were probably among the largest animals in October. The average weight decreased from 109.3 mg in October to 68.7 mg in December. The two March worms averaged 89.2 mg (Roe, 1971 for details of each month).

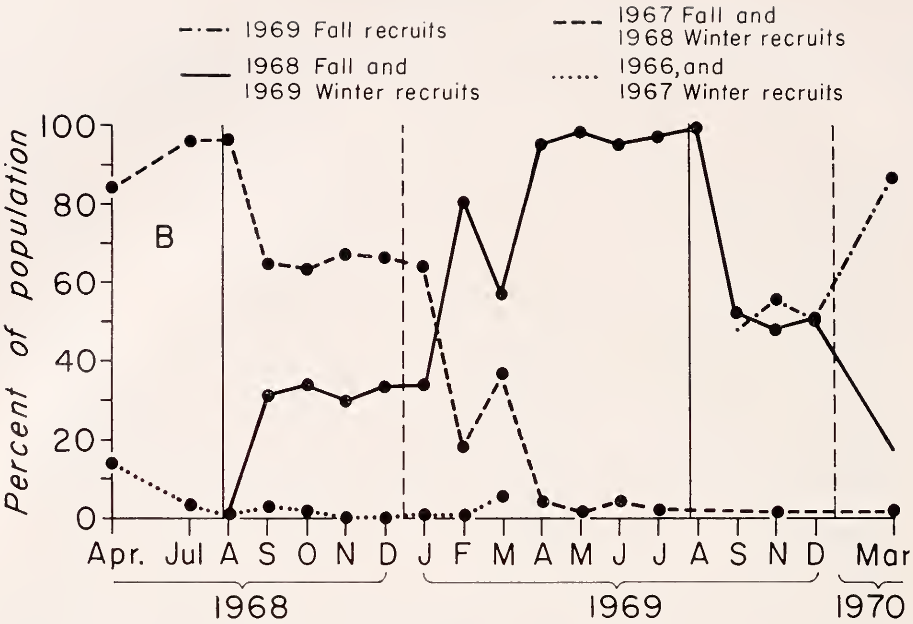


FIGURE 3. Per cent of Garrison Bay *Paranemertes peregrina* population comprised by each year class. Vertical solid lines indicate periods of recruitment and increased density. Vertical dashed lines indicate periods of adult mortality.

Recruitment and age classes. Young worms produced at the peak spawning periods of April (Garrison Bay) and June (Snug Harbor) are apparent in the fall population. Worms produced during minor fall and winter spawning are easily seen in spring. Since the young are usually lighter in color than adults, the per cent of the population that is light-colored can be used as an estimate of recruitment. At Garrison Bay small light animals comprised 26% of the population in September, 1968, and about 22% in October, 1969. At Snug Harbor recognizable (small, light) young constituted 6.1 and 8.4 per cents for 1968 and 1969, but the Snug Harbor population also had many small, dark members. Since it was difficult to determine with certainty the ages of the small, dark worms, although in the same size classes as the recognizable pale young, these figures are probably low (see also Roe, 1971).

Juveniles gradually get darker as they grow, and by March or April it is difficult to distinguish them from adults of previous years. Figure 3 shows the proportion of the Garrison Bay population comprised by each year class, from study of nemertean growth rate in the laboratory and that of young nemerteans in nature (see also Roe, 1971).

Life span and mortality. If they survive larval settlement, almost all members of mud flat populations of *P. peregrina* live about 1.5 to 1.75 years. Worms produced in the spring probably reproduce the following spring; and those produced in winter, the following winter. If a worm spawns more than once, it is probably one that has outlived the average life span. Indeed, at Snug Harbor there is a refuge near the −0.2 m tide mark where several large, old worms reside, and most worms 15 cm and longer seen in November–December were ripe. Light colored, small, young but ripe worms were also seen at Garrison Bay in the fall.

Mortality of *P. peregrina* was observed in nature only on very hot days when the lower low tide was just past noon; then only a few nemerteans were dying. Some animals also die after spawning. Size-frequency analyses (Fig. 1A, B) for the Garrison Bay population show sudden decreases in the numbers of large worms in fall or winter. Causes for these mortalities are not known. The gastropod *Aglaja diomedea* was observed to prey on *P. peregrina* (see also Gonor, 1964). However, *Algaja* is only large and common in summer when nemerteans are also large. Even the largest *A. diomedea* cannot eat an average sized adult *P. peregrina*; it can only bite off a piece of tail. *Aglaja* is also active at high tide, when the nemerteans remain underground. It is possible that *A. diomedea* has an effect on the numbers of small *P. peregrina* recruited, but it seems unlikely that it has much effect on the adult population. At Garrison Bay 2.7% of the *P. peregrina* population consists of worms with regenerating posterior ends; at Snug Harbor it is 5.1%, and at Edmonds, 3.5%. These percentages are biased downward, as regeneration was not always recorded, but the numbers of regenerating worms are few. *Aglaja* does not occur at Edmonds.

Another possible cause of mortality is parasitism by gregarines, although parasitized nemerteans appeared healthy. Twenty-six per cent of the Garrison Bay population had gregarine cysts in the faecal material. At Snug Harbor the average infection percentage determined from faecal analysis was 6.3%; and at Edmonds, 7.95%. In February, 1969, small pieces of gut tissue dissected from 17 Garrison Bay animals showed 88.2% were actually infected. Studies on the gregarine life cycle or on how nemerteans become infected were not done.

Population density. From numbers of nemerteans in twelve $\frac{1}{8}$ m^2 mud samples, it was estimated that 97% of the worms are active during the peak activity period of low tides in good weather during night tides at Garrison Bay. The total number/m^2 for Garrison Bay night tides is estimated per month as average density at low tide/0.97 (Fig. 4). In daytime summer low tides, ten samples of sieved mud yielded four inactive *P. peregrina*; two nemerteans were active in these areas. Because numbers were both low and variable, I was unable to determine with confidence the total number/m^2 for summer months. In spring, when lower low tides are in the afternoon, Garrison Bay nemerteans are not seen on the mud surface at low tide. By June, when these tides are in early morning, large (adult) worms are again seen in abundance (about 3/m^2). It is concluded that the nemerteans remain under the surface in spring since animals in June are adult size (Fig. 1B) and since *P. peregrina* does not usually move great distances (see section below on movements).

At Snug Harbor 70% of the nemerteans seen during night low tides (14, $\frac{1}{8}$ m^2 samples) and 50% in daytime low tides (32, $\frac{1}{8}$ m^2 samples) were estimated to be active during peak activity periods of these tides, with total number/m^2 each month being the average density at low tide/0.7 (night tides) or /0.5 (day tides) (Fig. 5). The summer estimates of the percentage of active worms may be low, as mud was sieved on days when few worms were active. These irregularities should be

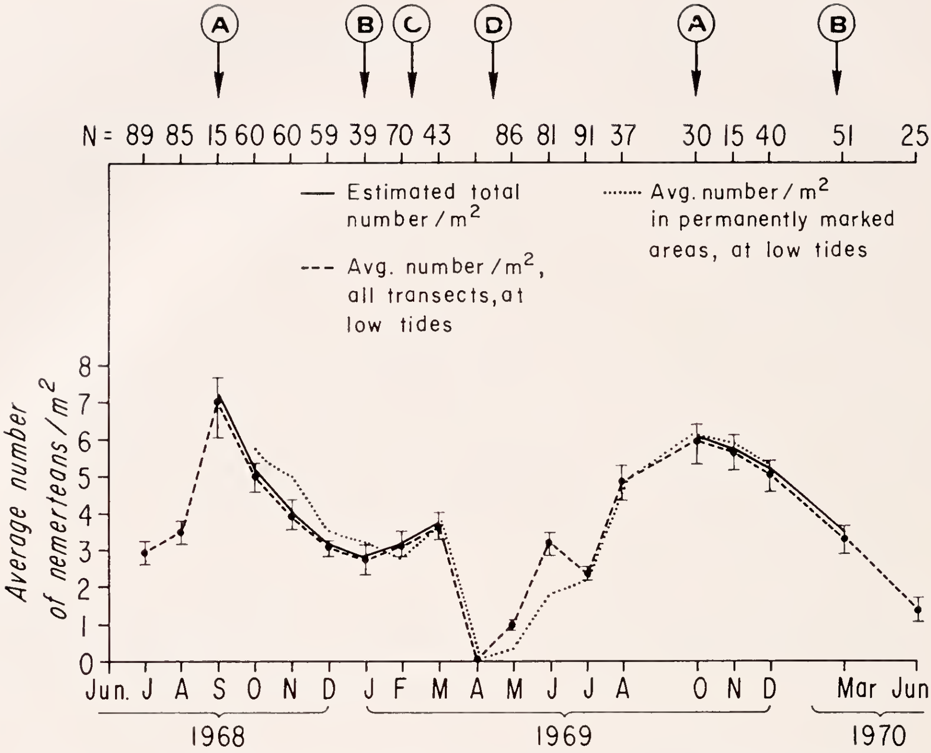


FIGURE 4. Mean densities (number/m^2) of *Paranemertes peregrina* at Garrison Bay. A) shows fall recruitment of juveniles from eggs spawned the previous spring; B) major mortality period of adults; C) spring recruitment of juveniles from eggs spawned the previous fall; and D) low densities due to animals staying under the mud surface, not mortality. Vertical lines along dashed line are ± one standard error from the mean. N is the number of 1 m^2 quadrats in which *Paranemertes* numbers were counted. Estimates of total density were calculated only for September 1968 to March 1969.

remembered during consideration of density information. At Edmonds only the numbers of active worms were counted (Roe, 1971).

At Garrison Bay (Fig. 4) and at Snug Harbor to some extent (Fig. 5) densities were highest in late summer and in fall, when both adults of the previous year and recruits from the previous spring or summer spawning seasons were present. Adults die in late winter (Fig. 3) reducing densities (Figs. 4, 5), but winter recruits add slightly to the populations then (Fig. 1A, B, small individuals seen in spring). At this time there is also replacement of the adult population by the present year's recruits, themselves reaching adult size (Fig. 1A, B).

Spring and summer are the most stable periods within each year with respect to density (Fig. 4); populations consists mainly of a one-year class (Fig. 5) and there is little mortality then. At Edmonds, such patterns were not determined. The number of active nemerteans depends strongly on weather conditions. Density measures in this higher intertidal area fluctuated too much for patterns to be

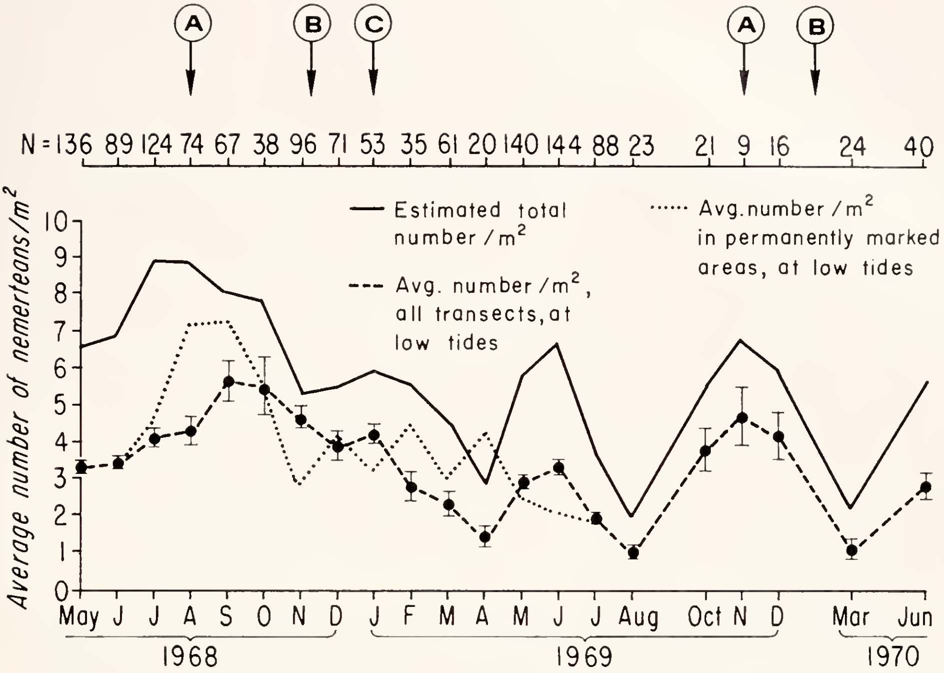


FIGURE 5. Mean densities (number/m²) of *Paranemertes peregrina* at Snug Harbor. A) shows fall recruitment of juveniles from eggs spawned the previous summer; B) major mortality period of adults; and C) spring recruitment from fall spawning. N is the number of 1 m² quadrats in which *Paranemertes* numbers were counted. Vertical lines along dashed lines are ± one standard error from the mean.

seen. At Snug Harbor, life history and densities are similar to those at Garrison Bay (Figs. 2, 5; Roe, 1971) but show more fluctuations.

Prey densities

Platynereis bicanaliculata. An annual, *P. bicanaliculata*, has highly synchronous spawning periods. A few spawned in June, especially at Garrison Bay; but most of both the Snug Harbor and Garrison Bay populations spawned near August 1 in both 1968 and 1969, at which time there was almost a complete turn-over of each. Young specimens of *P. bicanaliculata* settle out of the plankton in about three weeks, 4 mm or less in length. They quickly grow to about 10 mm long, and over-winter at this size. In spring they grow to adult size (Figs. 6A, 7A; Roe, in preparation).

Since *P. bicanaliculata* is the major prey organism of both mud flat *P. peregrina* populations, densities of this species were most carefully and frequently determined. Figures 6B and 7B show densities through time. There are not such strikingly linear decreases in numbers at Snug Harbor as at Garrison Bay. When samples were divided into those with and those without algae, trends showed that *P. bicanaliculata* tends to be more abundant in algal covered areas than in bare mud,

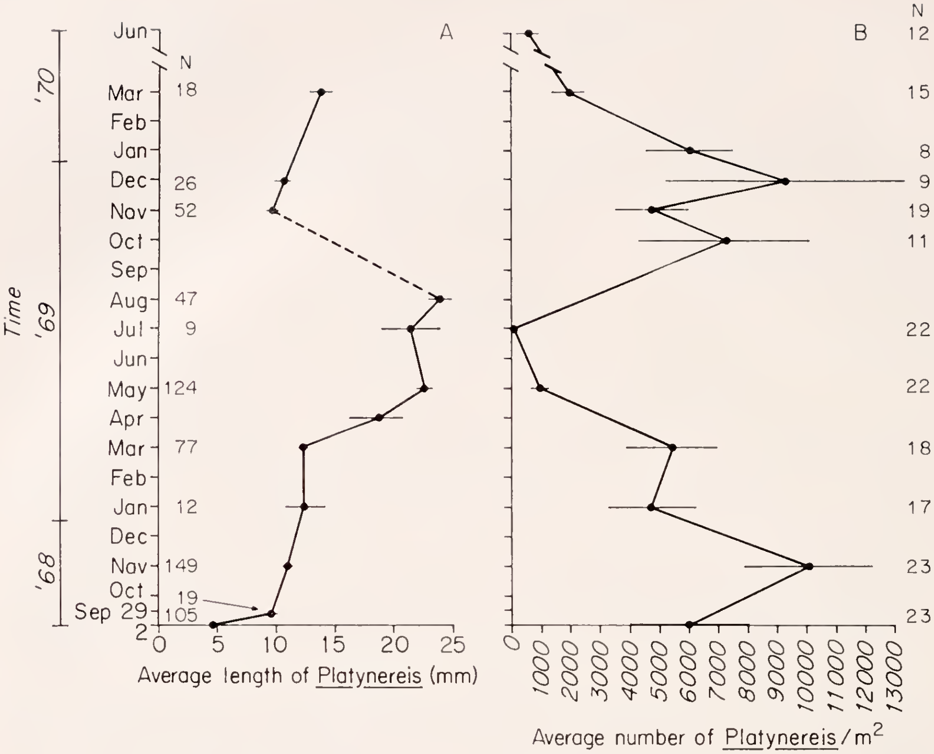


FIGURE 6. A) shows the average size ± one standard error of *Platynereis bicanaliculata*, September, 1968 to March, 1970, at Snug Harbor. N is the number of worms measured; B) mean number ± one standard error of *Platynereis bicanaliculata*/m^2 at Snug Harbor. The overall average number of *Platynereis* per m^2 is 3218. N is the number of samples taken. In August, 1969, young worms were collected and measured, but density was not determined.

although the numbers of samples in the two categories usually became too small to show significant differences (Roe, 1971). It was concluded that algal patchiness at Snug Harbor, not present at Garrison Bay, can account for the fluctuations. *P. bicanaliculata* is abundant in fall in both mud flats, with numbers decreasing until summer (Figs. 6B, 7B).

Other prey in mud flats. Densities of *Armandia brevis, Nereis vexillosa,* and spionids were estimated from fewer samples than *Platynereis*; the estimates of average density thus do not reflect the patchy distribution of the three species. At Garrison Bay the average number/m^2 of spionids in 41 samples over four months was 76.5, the average number of *A. brevis* in 75 samples in seven months was 143, and the average number of *N. vexillosa* in 49 samples in eight months was 3.3 (Roe, 1971 for data for each month). At Snug Harbor averages/m^2 were: *Scolelepis fuliginosa*, 119 in 87 samples over three months; *A. brevis*, 313 in 112 samples in seven months; *N. vexillosa*, 21.5 in 46 samples over eight months. At Edmonds, densities were difficult to compare since large barnacles provide fewer (but larger) cracks to live in than do small ones, and different polychaetes occupy each type of

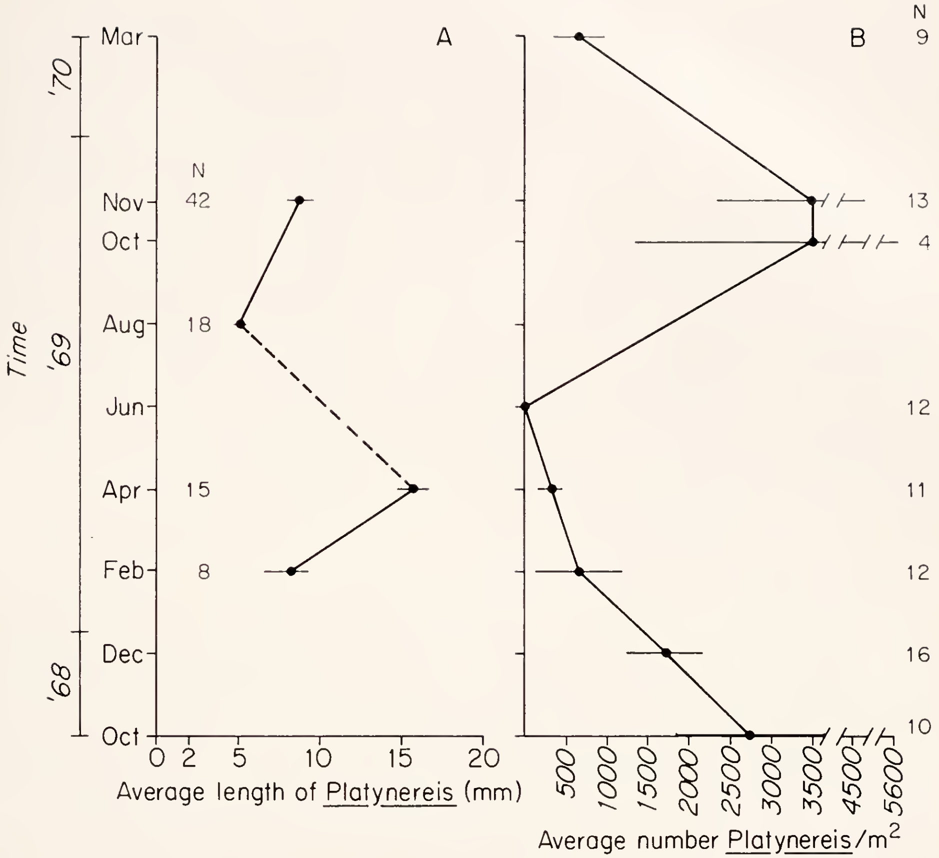


FIGURE 7. A) shows the average size ± one standard error of *Platynereis bicanaliculata* February, 1969 to November, 1969, at Garrison Bay. N is the number of worms measured; B) mean number ± one standard error of *Platynereis bicanaliculata*/m^2 at Garrison Bay, October, 1968 to March, 1970. N is the number of samples. All samples except June, 1968 (100 cm^2) were 20 cm^2. Overall average number of *Platynereis*/m^2 is 815.6. In August, 1969, young nereids were collected and measured but density was not determined.

crack. Table II shows the number of prey/ml of barnacles and mussels. No polynoids were found in the barnacle and mussel samples for prey; however, 28 were eaten by specimens of *P. peregrina* in two years. Low densities of nereids are valid. Densities of *Paleanotus* are underestimates because they were not counted for several months and because they are difficult to see.

Feeding by Paranemertes peregrina

Feeding at low tide. P. peregrina is active soon after being exposed by the receding water during lower low tides: seeking out, capturing, and eating polychaetes.

Nemerteans were collected immediately after leaving their burrows as the receding tide uncovered the mud flat. Faeces of these worms were collected, as well as

Table II

Estimated number of prey/ml of barnacles and mussels at Edmonds.

Substratum	Prey	Samples			
		Number of samples	Volume of samples, ml	Number of prey collected	Average number per 1,000 ml substratum
Large barnacles	Syllids	10	10,125	222	21.9
	Boccardia sp.	9	9,625	200	20.8
	Paleanotus chrysolepis	2	950	5	5.3
	Nereis vexillosa	1	10,125	1	0.1
Medium sized barnacles	Syllids	4	1,950	43	22.1
	Boccardia sp.	4	1,950	311	159.5
	Paleanotus chrysolepis	1	250	12	48
	Nereis vexillosa	1	1,950	3	1.5
Small barnacles	Syllids	8	4,300	169	39.3
	Boccardia sp.	8	4,300	608	141.4
	Paleanotus chrysolepis	1	50	2	40
	Platynereis bicanaliculata	1	4,300	1	0.2

faeces of worms collected about 1.5 hours after being uncovered by the receding tide, giving them time during the interval to feed. A significantly higher proportion of the latter had succeeded in feeding: at Snug Harbor, 28 had fed soon after being exposed, 120 had not; 96 had eaten and 77 had not after 1.5 hours exposure, $\chi^2 = 44.99$, $P < 0.005$. At Garrison Bay, five had fed and 21 had not soon after being exposed; seven had and 17 had not fed after 1.5 hours exposure, $\chi^2 = 0.660$, $0.5 < P < 0.25$. At Edmonds two had fed and 13 had not versus 15 feeding and 19 not feeding after 1.5 hours exposure, $\chi^2 = 4.35$, $P = 0.05$.

On the permanently marked areas at high tide periods when the substratum was under water, very few nemerteans were ever seen on the surface: an average of 0.01 worms was active/m^2 at Garrison Bay and an average of 0.03/m^2 was active at Snug Harbor. Even if these averages are multiplied by 23, the average number of hours the mud flats are under water per day, densities are only 0.23 and 0.69 worm/m^2, far lower than low tide densities (Figs. 4, 5). No worms were seen while diving at Edmonds. The nemerteans could be burrowing beneath the mud and capturing food, but in light of their "homing" behavior (see below), it is thought that they move little, unless disturbed, between exposure at low tides.

The artificial mud flat, run for ten months without "low tides," showed that the 16 nemerteans in the experiment shrank. One large worm decreased from 570 mg weight and 475 mm^2 in area to 226 mg and 142.5 mm^2 from October to April; a second decreased from 638 mg and 388.5 mm^2 to 283.5 mm^2 from October to March (not weighed in March). One group of smaller nemerteans decreased from average weights of 140 mg and average areas of 135 mm^2 between October and August to 35 mg and 44.2 mm^2. A second group decreased from average weights of 125 mg and average areas of 115 mm^2 to 36.3 mm^2 (not weighed) between October and August (Roe, 1971 for details). This decrease in size can be attributed to decreased feeding when there are no low tides, since many nereids were added to the aquarium periodically. However, *P. peregrina* has been observed feeding under water. It was concluded that in nature *P. peregrina* feeds primarily when exposed at low tide, although feeding can occur at other times, as in September and March, when few tides are low enough to expose the nemerteans.

Length of activity period. Many nemerteans are active during the first two hours after the tide recedes during months with daylight low tides, and during the first 2.5 hours in night low tides. Average density of active *P. peregrina* soon after the worms were exposed by the receding water during daylight tides was $3.3/m^2$, and after 1.5 hours, $1/m^2$; at night in 1.5 hours, densities decreased from $4.5/m^2$ to $2.8/m^2$. (Counts were made in the same square meters earlier and later.) Activity is modified considerably by weather. Rain, snow, and strong afternoon sun reduce the numbers of worms out and the length of time any one worm stays out. At Snug Harbor (one night in November) density was $3.9/m^2$ before it started raining, $1.4/m^2$ shortly after; density at Garrison Bay in March was $4.1/m^2$ after dark, and only two nemerteans were seen on the whole mud flat during two afternoon low tides. March night tide and afternoon densities at Snug Harbor were 3.2 and $0.8/m^2$, respectively.

Movements. Recaptures of 48 nemerteans dyed with neutral red and put into three marked 1 m^2 areas in September, 1968 at Garrison Bay showed: October, 32 recaptured, 67%; November, 12 recaptured, 25%; December, 5 recaptured, 10.4%; January, 2 recaptured, 4.2%; February, 1 recaptured, 2%; April, 1 recaptured, 2%; May, 1 recaptured, 2%.

At Snug Harbor 14 red nemerteans were put into a 1 m^2 plot in July, 1968. Results were: August, 6 recaptured, 42.9%; September, 2 recaptured, 14.3%; October, 1 recaptured, 7%; November, 1 recaptured, 7%.

Forty-nine red nemerteans were put into seven different marked 1 m^2 areas along a transect at Snug Harbor in September, 1968. Of these, 12 were recovered in five areas after one month; six were recovered in five areas after two months; two in two areas after three months; and four worms were collected in four respective square meters after five months. After five months, 4 out of 49 animals were still in the same meter where they had been placed.

After two weeks, experimental areas at Snug Harbor cleared of nemerteans had approximately 50% as many worms as before. Clearing experiments suggest that nemerteans will move into empty areas rather quickly. However, on several occasions it was casually noted that nemerteans stayed active longer and crawled more where many bootprints were left in the mud. Clearing an area disturbed not only that area but also the ground around it, perhaps causing worms in those areas to be more active than normal.

From Pielou, 1959, it is seen that when *P. peregrina* is active at low tide, individuals are randomly dispersed: at Snug Harbor the probability of random dispersion was $\gg 0.05$ in October, 1968; >0.05 in November, 1968; >0.05 in June, 1970 and in a second sample in June, 1970, $\gg 0.05$. At Garrison Bay the probability of random dispersion was $\gg 0.05$ in July, 1969 (see also Roe, 1971).

Individual feeding behavior. Feeding behavior of individual worms was observed both in nature and in artificial mud flats. The following is a synthesis of many observations. When the tide recedes at the beginning of a low tide period, the nemerteans emerge from their burrows and search for food. Most worms are out about 15 minutes after the water leaves an area (average time in 30 laboratory observations, 13.9 minutes). In the artificial mud flats (18 observations) worms crawled an average of 30 cm in 18 minutes in search of food. There is much variation in the distances traveled and speed of crawling.

P. peregrina searches haphazardly until it comes upon a nereid or a fresh nereid trail or tube. When contact is made with a trail it crawls along the trail, not always in the same direction as the nereid. It frequently loses the prey even if it comes upon the prey itself. The swimming-flipping escape response of nereids (Roe, 1970) is quite effective; contact with the nereid causes a flurry of motion, destroys the trail, and the nemertean sometimes loses the prey. *P. peregrina* was observed a few times to lift its head, swinging it back and forth across a trail. This behavior suggests that close-range chemoreception helps keep the nemertean oriented. *P. peregrina* has no distance chemoreception to aid in prey detection; it must bump into the prey or immediate surroundings of the prey with its head before it recognizes prey as prey. In the artificial mud flats *P. peregrina* spent an average of 17.8 minutes in random crawling before finding a nereid trail, tube, or nereid, and another 23.7 minutes poking its head down worm tubes and chasing prey organisms, for a total search time of 41.5 minutes. Search time was terminated when the feeding process (Roe, 1970) started or when a nemertean started home, having found no food.

When prey is found, feeding takes only a few minutes. In the artificial mud flats average feeding time was 8.3 minutes; in nature, 7.3 minutes. It takes longer if a *P. peregrina* goes into a *P. bicanaliculata* tube to feed, and it takes much longer (over an hour) if one tries to eat a nereid much larger than itself.

P. peregrina seems able to eat only polychaetes that are at or very near the surface of the mud or those that have broad tubes. Maldanids, oweniids, lumbrinerids, and other abundant polychaetes having deep, vertical tubes with small diameters were not eaten by the nemertean.

After feeding, *P. peregrina* usually backs up a few centimeters along the mucus trail it made during the search for food; then it turns around and crawls on the trail until it reaches its burrow, where the nemertean disappears underground. Most individuals return to their former burrows after each meal; they only make new burrows if part of the mucus trail was destroyed and they cannot find their way home.

The total time spent out at low tide looking for food, eating, and returning home averaged 41 minutes in the artificial mud flats and 43 minutes in nature for individual worms (not always the same observations as studied for crawling and searching).

Prey species and diversity of diet. Food preference experiments (Roe, 1970) indicated that behaviorally *P. peregrina* prefers to eat nereids over other polychaetes. (In this paper the word *preference* refers to what animals ate most often when given choices in laboratory experiments of Roe, 1970. The word *selectivity* refers to what animals actually eat in nature.) Faecal analysis of worms in nature also showed that nereids are the major prey in areas where they are available (Table III).

Specimens of *P. peregrina* at both Snug Harbor and Garrison Bay mainly eat nereids throughout the year (Fig. 8; Roe, 1971). In all three populations there is

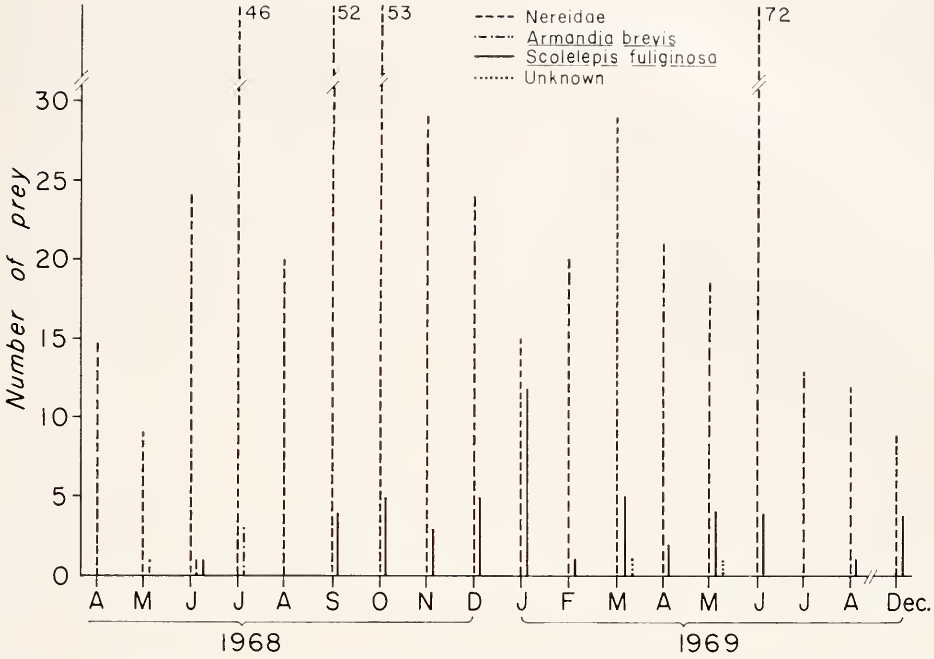


FIGURE 8. Numbers of each prey eaten by Snug Harbor *Paranemertes peregrina,* April, 1968 to December, 1969.

a significant increase in feeding on spionids during the winter: at Snug Harbor between September and April they ate 39 spionids, 234 other prey; between May and August they ate 12 spionids, 257 other prey; $\chi^2 = 15.34$, $P < 0.005$. At

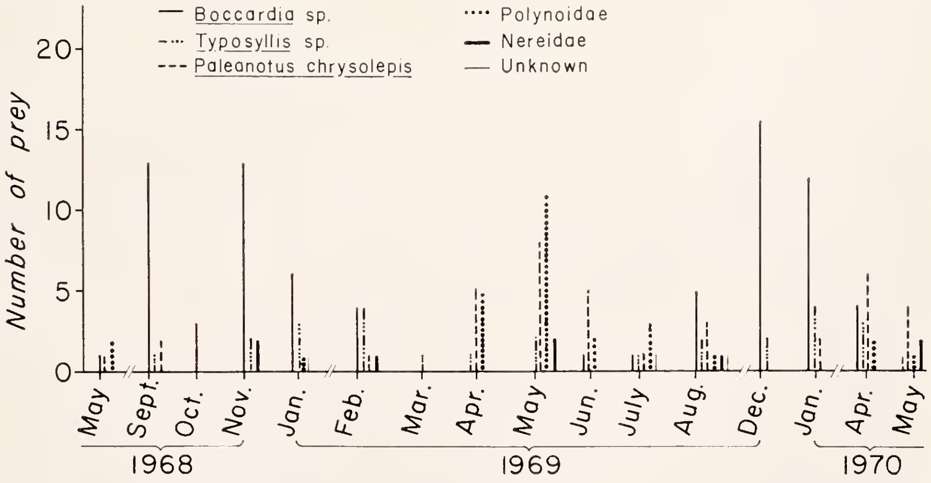


FIGURE 9. Numbers of each prey eaten by Edmonds *Paranemertes peregrina,* May, 1968 to May, 1970.

TABLE III

Per cent of total diet comprised by each prey species.

Prey species	Snug Harbor	Garrison Bay	Edmonds
Platynereis bicanaliculata	80.0	68.4	1.6
Nereis vexillosa	2.6	3.5	2.7
Nereids*	6.7	4.1	
Total Nereidae	89.3	76.0	4.3
Spinoids	9.4	18.1	43.1
Ophiodromus pugettensis	0	5.1	0
Armandia brevis	0.9	0.9**	0
Paleanotus chrysolepis	0	0	20.8
Polynoids	0	0	15.3
Typosyllis	0	0	14.7
Unknown*	0.4	0	1.6
Total	100.0	100.1	99.8
Total number prey	542	216	183

* In these samples there were too few setae or some other reason which made species identification unreliable.

** These setae were tentatively identified as *Armandia*; they might conservatively be placed under "unknowns."

Garrison Bay between October and February they ate 31 spionids and 80 other prey; between March and September, 6 spionids, and 91 other prey; $\chi^2 = 16.73$, $P < 0.005$. At Edmonds between September and February they ate 67 spionids and 27 other prey; between March and August they ate 12 spionids and 78 other prey; $\chi^2 = 62.99$, $P < 0.005$.

Small numbers of *Armandia* and *Ophiodromus pugettensis* are also eaten at Snug Harbor and Garrison Bay, especially in summer (Fig. 8; Table III). At Edmonds *Paleanotus* and polynoids replace spionids as the major prey during summer. Syllids (*Typosyllis* sp.) are eaten throughout the year (Fig. 9; Table III) at Edmonds.

Using Pielou's (1966) diversity index, it was found that at Edmonds, prey-species diversity is significantly higher in summer ($H' = 1.39 \pm 0.02$) than in winter ($H' = 0.89 \pm 0.04$) at the 95% level. At Garrison Bay prey-species diversity is essentially the same in summer ($H' = 0.66$) and in winter ($H' = 0.64$). At Snug Harbor summer months show a significantly lower diversity ($H' = 0.30 \pm 0.01$) than winter months when many spionids are eaten ($H' = 0.44 \pm 0.03$) at the 95% level. Prey-species diversity is higher at Edmonds than at Garrison Bay and higher at Garrison Bay than at Snug Harbor.

Electivity. Electivity coefficients (Ivlev, 1961) of all prey species whose relative densities in nature are known indicate that at Snug Harbor and Garrison Bay specimens of *P. peregrina* eat nereids about as often as they come into contact with them (electivity coefficient for Snug Harbor, +0.01; for Garrison Bay, +0.10), that they select spinoids (+0.70 and +0.51), and that *Armandia* is usually passed by (−0.81 and −0.85). At Garrison Bay *Ophiodromus* is also usually passed by (−0.61). Actually, nereids are probably sought out soon after *P. pere-*

grina is uncovered by the receding water of low tide when the nemerteans are hungry but are more likely than other species to be passed up later in the low tide period when the nemerteans have already fed, simply because nereids are so much more abundant than other species.

At Edmonds, *Paleanotus* (+0.16) and nereids (+0.87) are selected, *Typosyllis* (−0.24) is not, and *Boccardia* is eaten as it is encountered in winter (−0.005) and in cracks between small barnacles where it is very common (−0.01) and is eaten less often than encountered in cracks of large barnacles (−0.32). The high electivity for *Paleanotus* is probably a function of underestimating *Paleanotus* densities. Four feeding experiments suggested that *Paranemertes* will not often accept *Paleanotus*.

Natural history and escape responses. Although electivity shows mathematically what prey *P. peregrina* selects, this coefficient is not nearly sufficient to explain feeding patterns. Behaviorally, *P. peregrina* strongly prefers nereids to all other prey (Roe, 1970), yet electivity values of mud flat populations indicate little selectivity. Natural history of prey, and prey behaviors, as escape responses, are important in determining prey availability. Densities alone are insufficient for understanding availability.

At Snug Harbor during low tides on hot summer days specimens of *P. peregrina* were feeding almost exclusively on *Armandia brevis*. (In all observations on feeding 21 specimens of *A. brevis* were seen being eaten, plus four attempts by nemerteans to eat *Armandia*.) However, *A. brevis* is less than 1% of the nemertean diet there (Table III). *A. brevis*, a normally active animal, has a highly effective flipping, swimming escape response to touch. Only on hot summer days when the low tide is in early afternoon can *P. peregrina* catch *Armandia*, sluggish from heat stress. Availability of this prey is, therefore, temporally highly patchy.

At Garrison Bay *P. peregrina* caught and ate juvenile *Ophiodromus* in summer. *Ophiodromus* as an adult makes *P. peregrina* turn another direction upon contact and not even attempt to eat it. Juveniles are only present in summer.

P. peregrina tries to feed on *Nereis vexillosa* about as frequently as on *Platynereis*. However, faecal analysis shows that *N. vexillosa* comprises only 2.6% of the food actually eaten versus 80% being *Platynereis* (Table III, Snug Harbor). Food preference tests (Roe, 1970) showed that it is harder for the nemerteans to finish the feeding process with *N. vexillosa* as prey. Present studies confirm this; over half of the observations on feeding in nature were not completed (*P. peregrina* ate nine nereids but did not complete feeding on 11 others in 20 observations). *N. vexillosa* has several escape mechanisms making it less available. It escapes in size from *P. peregrina* twice in its life span. When tiny (3 to 8 segments), it is potential prey for one to two month old nemerteans only; adult nemerteans do not notice prey this small. However, it quickly grows to a size that the slower growing young nemerteans cannot handle. During the first year of the nereid's life span, after it reaches a size to be noticed by larger nemerteans, it is a size that *P. peregrina* can eat. In the second year (Roe, in preparation) the nereids grow too large in diameter for the nemerteans to ingest. These large nereids also usually recover from the paralytic effects of the toxin injected into them during unsuccessful attempts by *P. peregrina* to feed on them. *N. vexillosa* also displays the typical swimming escape response of nereids to *P. peregrina* (see Roe, 1970). This escape response is in effect throughout life; a three-segment *Nereis* showed response to touch by a larval *Paranemertes* 0.3 mm in length. Large nereids show aggressive behavior toward specimens of *P. peregrina*; instead of just trying to escape, they sometimes bite their attackers. If a nereid bites a nemerteans's proboscis, the nemertean cannot draw the proboscis back within its rhynchocoel, and feeding, if successful at all, takes much time. Finally, *N. vexillosa* tends to become active at the end of a low tide period when water is beginning to cover the mud. As this is the time when nemerteans have already fed and are returning home, the nereid activity period becomes a protective device.

Platynereis bicanaliculata is the major prey of *P. peregrina* where it is common. It is in the preferred food family, and it has only the flipping-swimming escape response. This behavior is much more effective at high tide, and *P. peregrina* usually succeeds in capturing *Platynereis* at low tide. It is suggested that *P. peregrina* has evolved to do most feeding when low tides expose the substratum because the nereid escape response is less effective when the nereid can only flip a few centimeters than when it can swim away. *P. bicanaliculata* is of a size range suitable for eating throughout the year (Figs. 6A, 7A). It was never observed to fight back when struck by a nemertean. Syllids are difficult for *P. peregrina* to ingest even though they are small (Roe, 1970).

The difference in feeding in all three populations on spionids in winter and summer must be due in part to behavioral differences that make spionids more available in winter. Densities of spionids are roughly the same in both seasons (my observations for Snug Harbor, confirmed by S. A. Woodin, Johns Hopkins University, in personal communictaion), and nemerteans strongly preferred nereids in food preference tests run during winter (Roe, 1970).

Time taken for passage of food through the gut. Defecation usually occurs 18–20 hours after feeding (see also, Roe, 1970; Gibson, 1970), so faecal samples obtained from worms of a given tidal period represent feeding that occurred during that same tide.

Feeding time through a tide series. Worms were collected every day over four to six days, when lower low water level uncovered them; presence or absence of faeces of worms collected on early or on later days during a low tide series showed no significant differences in numbers of worms feeding at the beginning and at the end of the tide series (Snug Harbor: days 1–2, 19 fed, 69 did not; days 5–6, 9 fed, 45 did not; $\chi^2 = 0.512$. Edmonds: days 1–2, 7 fed, 11 did not; days 3–4, 8 fed, 11 did not; $\chi^2 = 0.037$). It was concluded that in general *P. peregrina* feeds about every day if it can find prey.

Feeding per cents through the year. At Snug Harbor an average of 66.5% of the population ate per low tide during night low tides (September to January, 149 of 224 worms collected had eaten). During daylight low tides of April to July an average of 33.1% ate during a low tide (199 of 401 worms collected). At Garrison Bay during night low tides of September to January an average of 44.5% (85 of 191 collected) ate per low tide. In daylight tides of April to July an average of only 16.4% (46 of 279 collected) ate per low tide. At Edmonds daytime and night patterns were less striking; the overall feeding rate was 38.6% (183 of 474 worms collected). (See Roe, 1971, for details for all 3 populations.)

Since nereids have jaws, the number of prey eaten per nemertean can be determined from faecal analysis. At Snug Harbor during night low tides of September to March an average of 19.7% (42 of 213 worms collected), and during daylight tides of April to August 12.3% (19 of 155) of the nemerteans which had fed had eaten more than one prey item while exposed at a given low tide. At Garrison Bay during night tides of August to March an average of 11.4% (16 of 141 collected), and during daylight tides of April to July, none of the feedings were multiple. Comparable data are unavailable for the Edmonds population since few prey had markers to distinguish between two similar-sized prey of the same species.

Maximum feedings rate. From laboratory feeding experiments in which 12 nemerteans were fed as many individuals of *P. bicanaliculata* as they would eat per day (experiments lasted varying lengths of time) and information derived from the numbers of nereids eaten per day by the three juveniles grown in the laboratory for 6–18 months, it was determined that an individual *P. peregrina* eats 0.83 to 0.95 polychaete per day, if prey is available.

Size of prey eaten. More detailed studies of feeding on *P. bicanaliculata* were done since prey sizes could be determined by measuring jaws in faeces. A linear regression of nereid length to jaw length is significant (length = 31.9 jaw length -5, $F = 249.27$, $P < 0.005$). During most of the year there was no significant correlation or linear regression between nemertean size and the size of *P. bicanaliculata* eaten. Data were divided into seasons corresponding to the three size categories of *P. bicanaliculata* (Fig. 6A). Results show: late August to early September, $r = 0.803$, $N = 12$, $P = 0.001$; October to March, $r = 0.201$, $N = 72$, $0.1 > P > 0.05$; April to July, $r = 0.084$, $N = 67$, $P > 0.1$. In August a few large individuals of *P. bicanaliculata* are still available as food, and new recruits are small enough (about 4 mm long) to escape notice of larger nemerteans. So for a brief time, one sees large nemerteans usually eating last year's adult prey and nemertean recruits eating nereid recruits. But, by early October, no adults of *P. bicanaliculata* remain and recruits have grown large enough (10–12 mm, Fig. 6A) for all nemerteans to eat.

P. peregrina tended to eat more of the larger prey than the smaller ones during fall and winter when nereids are generally small. Significant differences between sizes eaten and sizes available were determined using Student's *t*-test, with the following results: early September, 1968, $t = 2.309$, $0.05 > P > 0.025$; late September, 1968, $t = 0.190$, $0.10 > P > 0.05$; November, 1968, $t = 2.222$, $0.05 > P > 0.025$; January, 1969, $t = 1.742$, $0.10 > P > 0.05$; March, 1969, $t = 3.518$, $0.001 > P$; April, 1969, $t = 0.848$, $0.5 > P > 0.4$; May, 1969, $t = 1.306$, $0.2 > P > 0.1$; December, 1969, $t = 1.123$, $0.3 > P > 0.2$.

Individuals of *P. peregrina* that had eaten more than one prey during a low tide were not significantly larger than those that had eaten only one prey. *T*-tests of the two groups of nemerteans gave the following results: late August to early September (a 2–3 week period), $t = 0.60$, $0.6 > P > 0.5$; late September to March, $t = 0.35$, $0.8 > P > 0.7$; April to early August, $t = 0.02$, $0.9 > P > 0.8$.

The average size of *P. bicanaliculata* eaten in multiple feedings was not significantly different from the average size eaten in single feedings. Jaw sizes of nereids from multiple feedings and from single feedings were compared using *t*-tests: late August to early September (a 2–3 week period), $t = 1.17$, $0.3 > P > 0.2$; late September to March, $t = 0.834$, $0.5 > P > 0.4$; April to early August, $t = 1.75$, $0.1 > P > 0.05$.

TABLE IV

Estimated number of each species eaten by Paranemertes peregrina *per square meter per year and per cent of each population consumed.*

	Snug Harbor		Garrison Bay		Edmonds*	
	Number	Per cent	Number	Per cent	Number	Per cent
Platynereis bicanaliculata	437.2	13.6	281.2	34.5	9.6	83.5
Nereis vexillosa	14.3	65.0	14.4	436.4	16.0	96.4
Nereid sp.	36.4	—	16.8	—	—	—
Total Nereidae	488	15.1	312.4	38.1	25.6	91.9
Spionidae	51.5	43.3	74.2	96.0	252.2	24.3** 3.2*** 3.6****
Armandia brevis	5.0	1.6	3.8	2.7	—	—
Ophiodromus pugettensis	—	—	20.9	—	—	—
Syllidae	—	—	—	—	86	7.9** 7.8*** 4.4****
Paleanotus chrysolepis	—	—	—	—	121.5	—
Polynoidae	—	—	—	—	89.5	—

* Numbers/1,000 ml were multiplied by 50 to get an estimate of numbers/m^2.
** In samples of large barnacles.
*** In samples of medium-sized barnacles.
**** In samples of small barnacles.

Estimated number of prey eaten by P. peregrina. The direct effect of *P. peregrina* on prey species is to reduce their numbers. The estimate of the average number of prey eaten by specimens of *P. peregrina* per m^2 per year was calculated to be 546.5 at Snug Harbor and 411.1 at Garrison Bay. At Edmonds the minimum estimate (585.1 prey/m^2/yr) was considered most correct (see Roe, 1971, for details and methods of calculating these figures). Table IV shows the number of individuals of each species eaten per year per m^2 and what per cent of the available prey population is eaten by the nemerteans per year. These figures are reliable for all spcimens of *P. bicanaliculata, N. vexillosa* at Snug Harbor, and spionids and syllids at Edmonds. Densities of spionids at Snug Harbor and *Armandia* at Snug Harbor and Garrison Bay are probably fairly correct.

DISCUSSION

Although life histories of two populations of this nemertean were similar, spawning periods were different in all three populations. Spawning must be associated with local events. Larvae of *P. peregrina* can survive at least eight weeks without food, or they can start feeding within a month after being spawned. One month old nemerteans indicated that they, like adults, show a strong preference for nereid prey. *N. vexillosa* spawns from March through August (Roe, in preparation), but the egg masses are patchily distributed and the three-segmented young quickly outgrow very young specimens of *P. peregrina*. *P. bicanaliculata* is probably the main food source for very young nemerteans; it is temporally predictable and abundant. The timing of nemertean spawning has probably evolved to coincide closely enough with its major prey's spawning that the tiny nemerteans find food sometime within their first two months of life. Some individuals of *P. bicanaliculata* spawned in late June at Garrison Bay, whereas nearly all at Snug Harbor did not spawn until August; nemerteans spawn later at Snug Harbor than at Garrison Bay. Spionid larvae are in the plankton throughout spring and summer and might also be a food source, especially for the young at Edmonds.

Feeding is a most important activity for any animal. For predators that pursue relatively large prey, the most adaptive strategy is to specialize when food is abundant but become less specialized when food becomes scarce (Emlen, 1966; Mac Arthur and Pianka, 1966; Ivlev, 1961).

P. peregrina is a pursuer, spending on average 18 minutes haphazardly searching for prey, and 24 more minutes in pursuit of prey or in poking its head down prey tubes.

This study of *P. peregrina* allowed predator strategy theories to be tested by comparing behavior of the same species in areas varying greatly in abundance of preferred nereid food. Snug Harbor had an average of 3240 nereids/m^2, Garrison Bay, 819/m^2, and Edmonds, about 28/m^2 (Figs. 6B, 7B; Table II). At Snug Harbor the preferred nereids constituted 89% of the diet, and there were two minor prey. At Garrison Bay nereids comprised 76% of the diet, and there were three minor prey groups. Spionids were relatively more important, comprising 18% of the total. And, at Edmonds, nereids comprised only 4.4% of the diet, with spionids increasing to 43% and diversity of prey increasing to five families (Table III).

At Snug Harbor a higher proportion of the nemerteans fed during low tide periods (overall feeding average of 42.8%), and a greater number of nemerteans ate more than one prey per low tide period (overall average of 16.9%) than at Garrison Bay (overall average feeding of 30.5%) with 9.6% being multiple feedings over the year. At Edmonds 38.6% of the nemerteans sampled during low tide periods had fed. From the proportion of nemerteans feeding, Snug Harbor appears to be the best of the three areas for *P. peregrina*. And, although prey are abundant at Edmonds, preferred prey are not; adult nemerteans there are far smaller than adults at either mud flat areas (Fig. 1A, B; Roe, 1971). It appears that the preference *P. peregrina* shows for nereids reflects a long evolutionary association with these prey with a correlated greater efficiency in capturing or assimilating them to other species. The Edmonds population may be food-limited in spite of a seeming prey abundance.

Seasonal feeding patterns exist within each population. At Snug Harbor prey-species diversity is significantly higher from September to March than April to August. This appears contradictory to prediction, as *P. bicanaliculata* averages 6000/m^2 in winter and only 260/m^2 in summer (Fig. 6B), and the per cent of *P. peregrina* feeding per low tide period in winter was greater (67%) than in summer (33%). However, *P. bicanaliculata* individuals are two to three times as large in linear dimensions in summer as in winter, and some are ripe females; both factors enhance the food energy per prey individual consumed in summer. Since all three populations of nemerteans did eat spionids in fall and winter, it appears that *P. peregrina* is to a limited extent an opportunist. Another suggestion of opportunism is the observation that *Armandia* was eaten in large numbers when the *Armandia* individuals were sluggish. *P. peregrina* may consume a less abundant, less preferred food in spite of an abundance of preferred nereids if the less preferred food becomes easily available.

The direct effects of predators on their communities are those they have on their prey populations (*e.g.*, Connell, 1961, 1970). Indirect effects are observed when changes in prey populations cause further alterations in community structure, changing abundances and diversity among organisms not directly affected by the predator (*e.g.*, Paine, 1966; Dayton, 1971).

Direct effects of *P. peregrina* are in lowering prey densities and in lowering reproductive potentials of prey species. From estimates of the feeding rate of *P. peregrina* and of prey densities, it was found that on mud flats *P. peregrina* eats 14–35% of the standing crop of *P. bicanaliculata* per year (Table IV). At Snug Harbor *P. bicanaliculata* is so numerous this 14% probably has little effect on reproductive capacity. At Garrison Bay, where nemerteans crop 35% per year, few nereids reach maturity; and even though each surviving female released thousands of eggs, recruitment was considerably lower than at Snug Harbor (Figs. 6B, 7B).

Whether *P. peregrina* has important indirect effects on its communities is a function of its prey; it is only when the major prey is the superior competitor for a basic resource that indirect effects of predation are strongly felt throughout the community (Paine, 1969).

At Snug Harbor, *P. bicanaliculata* is a major holder of the limited space available (Woodin, 1974). Significant reduction of nereid numbers allows larger numbers of *Armandia* and *Axiothella* (maldanid) to survive (Woodin, 1974). By heavy predation on *P. bicanaliculata, P. peregrina* can help maintain densities of other polychaetes in its communities. At Garrison Bay polychaete diversity is higher than that at Snug Harbor; this may in part be a result of the nemertean's cropping more of the nereid population there.

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SUMMARY

In Washington State, *Paranemertes peregrina* spawns in spring and summer, with minor spawning periods in fall. Juveniles from spring spawning are first easily seen the following fall, when the population consists of both adults and juveniles. Throughout winter adults die and juveniles mature, becoming ripe for the spring and summer spawnings. These adults die the following winter; the life span is thus 1.5 to 1.75 years.

P. peregrina feeds exclusively on polychaetes; at both mud flats *Platynereis bicanaliculata*, an annual, was abundant and the dominant prey organism.

Observations in nature and in artificial tidal flats constructed in the laboratory permitted study of foraging behavior and activity patterns. *P. peregrina* emerges from its burrow when exposed by the receding tide and searches haphazardly until it contacts acceptable prey. After contact, feeding takes seven to eight minutes. Then *P. peregrina* returns to its burrow by following its own mucus trail. Individuals often remain within a one meter radius for several months. Maximum feeding rate was determined in laboratory studies to be 0.8–0.95 prey individual per day.

In two intertidal mud flats and one rocky area, testing of predator strategy models and effects of predation on prey populations showed that in all three areas the percentages of nereids in the diets of *P. peregrina* were directly proportional to nereid density. Prey-species diversity was inversely proportional to nereid density. *P. peregrina* ate a significantly greater number of spionids during fall and winter than during spring and summer. *Armandia brevis* was also eaten by mud flat populations in summer, as was *Ophiodromus pugettensis* in one area. Polychaetes of five families comprised the diet of the nemerteans in the rocky area. Escape responses strongly affect prey availability. On mud flats *P. peregrina* tends to select *P. bicanaliculata* larger than the mean size available during fall and winter when *P. bicanaliculata* is small; larger nemerteans do not select larger prey than do small ones.

P. peregrina crops 14–35% of the *P. bicanaliculata* population per year, and probably somewhat higher proportions of *Nereis vexillosa* and spionid populations. At the location where predation on nereids was more intense, their recruitments were lower. By heavy predation on *Platynereis, P. peregrina* may help maintain densities of other polychaetes in its communities.

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CAN X-RAYS BE USED TO INCREASE LITTER SIZE IN THE MOUSE?

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Evidence that X-rays can sterilize gonads is abundant (Carter, Lyon, and Phillips, 1954; Casarett, 1956; Ingram, 1958; Jones, 1960; Mole, 1959; Nuzidin, Shapiro, Petrova and Kitaeva, 1955; Rugh, Deschner, and Grupp, 1960; Russell, 1958; Sanderson and Stearner, 1956), but there has also appeared in the literature some reports that low dose X-rays can stimulate ovulation, either to overcome sterility or to induce superovulation. Probably the first suggestion that X-rays could overcome amenorrhea or sterility in women was made by Fraenkel (1914). The next year Flatow made similar observations on the human female, but these were not reported until 1922. The idea was used in medical practice by Rubin (1926) and extensively by Kaplan (1938–1960), who published ten papers reporting direct ovarian X-radiation and occasionally supplementing it with X-radiation of the master reproductive gland, the pituitary, for some 3000 sterile women. He reported some 35% success in achieving pregnancies in women who had previously been diagnosed as being sterile, but multiple births were not significantly increased. He attributed his success solely to X-radiation. It was Asherman, however, who in 1952 suggested that simultaneous X-radiation of the ovaries and the anterior pituitary might be more effective than simply irradiating the ovary in overcoming sterility.

While no one can extrapolate data from another species to the human, and human data are rare and hard to come by in statistically significant numbers, it seemed in order to investigate this matter systematically on fairly large numbers of experimental mammals (mice) with varying exposures to whole-body X-rays, as well as at various times relating to the estrous cycle. Such a study is reported here.

Materials and Methods

Sexually mature female (CF_1) mice, three to four months of age, were exposed to whole-body X-radiation to a maximum of 200r, and over 8000 offspring were counted. This exposure is far below the LD/50/30 (50% lethal dose in 30 days) of 610r X-rays for this strain of mice. The exposures were single and ranged from 10r to 200r after which unirradiated but sexually mature males were added either immediately or after one week (as designated). The delayed mating was used to allow one estrous cycle, which was producing a mature irradiated ovum, to pass so that the irradiated ovum from the succeeding estrus would be fertilized. Should superovulation be evident, this would mean stimulation of an earlier stage in oögensis.

The radiation factors were: Westinghouse, Kilovolt peak 250, milliamperes 15, filters 0.44 tin, 0.25 copper, 1 aluminum with half value layer of 2.37 mm copper, distance 171.2 cm target specimen distance, rate 6.23r/minute using a Regato collimator. For the 100r–200r levels of exposure the following factors were changed: filters 0.5 copper, +1 aluminum, half value layer 1.37 mm copper, distance 1 meter target specimen distance, and rate 32.5r/minute.

The females selected for the various estrous stages by vaginal smears were X-radiated twelve at a time in a screen-pie tray through which the rays were free to pass and in which the mice were free to move about within the radiation field. Calculations of dose rate were made with the same supporting materials used during exposure. Following exposure they were placed twelve to a large animal cage, and two males were added to each box, either immediately in one series or after one week's delay in the other series. This ratio of one male to six females is adequate to keep all females pregnant since their estrous cycles range over a five-day period and are rarely simultaneous. Even so any sexually mature male can impregnate three females in any 24-hour period. The second series was delayed so that the ova in the immediate process of meiosis were not involved in the experiment, but rather those öocytes being prepared for the succeeding ovulations were exposed and fertilized. In both series the control females for irradiation were selected at random, without respect to estrous phase.

All females were sacrificed and dissected at late pregnancy, usually day 18 or 19, just before expected birth. To allow delivery would introduce an erroneous set of data, since mice often will destroy their newborn especially when one or more happen to be malformed. In sacrificing before birth we are able to determine the number of implantations, resorptions, anomalies, and apparently normal fetuses. Since it has been suggested (Hahn, *et al.*, 1964–1972; Valentine and Hahn, 1971; Ward and Hahn, 1967; Feingold and Hahn, 1972, 1973) that the phase of the estrous cycle might somehow be related to different susceptibilities to induced superovulation, a second experiment was conducted to expose female mice to 10r at each of the major phases of the estrus cycle: estrus, diestrus, and metestrus. Metestrus, as used herein, includes both pro- and post-estrus. A group of randomly selected females that covered the gamut of the maturation stages (Womack and Bogart 1969) was used as non-exposed controls. Since Pozhidaev (1960) reported that a high exposure of 600 r induced superovulation in rats, it is quite possible that at our lower levels of exposure of female mice there may have been few, if any, pre-implantation deaths. However, Harvey and Chang (1964) stated that exposure of hamster ovarian oöcytes in estrus to X-rays from 25r to 200r caused some ova to develop micronuclei in direct proportion to X-ray exposure, and that these micronuclei might be the cause of some pre-implantation deaths. Therefore radiation during estrus led to reduced litter size. The data presented here include all fertilized ova that were implanted. We are primarily concerned with the size of the litters, and the anomalies that X-rays to the pre-fertilized ova could produce. In a recent paper (Rugh and Budd, 1975), it is shown that even with 500r whole-body exposure of mice, including both ovaries, there was no statistically significant change in litter size nor any evidence of congenital anomalies, when such ova were fertilized by normal non-irradiated sperm.

RESULTS

The average litter size and percentage resorption in sexually mature female (CF_1) mice exposed to various levels of X-radiation are shown in Table I. The

TABLE I

Litter size and resorptions in mice exposed to various levels of X-radiation with immediate mating.

Condition X-rays in r	Pregnancies	Number offspring	Average litter size*	Per cent resorptions
Controls	112	1179	10.5	1.5%
10r	46	439	9.54	3.4%
20r	11	119	10.8	1.0%
30r	47	505	10.8	4.0%
40r	222	2403	10.8	0.2%
50r	48	510	10.7	6.0%
100r	40	463	11.5	4.0%
200r	40	391	9.8	7.0%

* Note absence of any trend with increasing exposure dose.

females were selected without regard to their stage of estrous, and mature males were provided immediately after irradiation for mating on a continuous basis so as to be available to all females when they were in estrus. Table II shows average litter size for similar females exposed under similar conditions, but with a one week delay before mating.

Among litters resulting from immediate post-radiation mating, the variation in percent resorptions over exposure levels was minimal, while variation in average litter size was within the range of normal experience for these mice. Neither variable exhibited any trend over the exposure range. Among mice mated one week after exposure (Table II), the average litter size is lower in the control group than in the irradiated groups, but the magnitude of the difference is small and not statistically significant.

With the prior knowledge (Rugh and Budd, 1975) that even 500r to the female mouse would not produce anomalies, nor alter fertility when the oöcyte (as part of the body) is exposed to X-rays, the above data represent an exploratory venture to determine which lower exposure to X-rays might alter the resorption rate or even induce some degree of superovulation. Neither occurred to any significant degree.

TABLE II

Litter size in mice exposed to various levels of X-radiation with one week delay before mating.

Exposure in roentgens	Number of litters	Percent resorption	Litter size	
			Mean	Standard deviation
0r	16	1.5%	9.75	2.14
10r	21	2.4%	11.38	2.84
20r	19	4.8%	10.47	2.06
30r	23	6.5%	10.65	3.01
50r	19	3.6%	11.53	2.06
100r	14	6.0%	11.43	1.28

Table III

Exposure to 100r of X-radiation at various estrous stages followed by immediate mating.

Estrous stage	Number of litters	Code*	Percent resorptions	Litter size		
				Mean	Standard deviation	Standard error
Control**	18	a, b	1.0%	10.00	2.72	0.64
Estrus	31	a	2.8%	9.16	2.68	0.48
Diestrus	53	b, c	3.0%	11.15	2.58	0.35
Metestrus	25	c	1.8%	11.72	1.57	0.31

* Code: Means with different codes are significantly different when tested at the 5% probability level ($P \leq 0.05$). Means with the same code letter are not significantly different.

** Control: Females chosen at random without respect to estrous stage.

The results of exposure to 100r of X-irradiation at the various estrous stages followed by immediate mating are shown in Table III. No statistically significant difference in average litter size occurred between groups exposed at diestrus and metestrus, but both groups had slightly larger mean litter size than those exposed at estrus ($P \leq 0.05$). The unirradiated control group, which probably included all stages of estrous was intermediate in mean size of litter and not significantly different from the groups irradiated at either estrus or diestrus.

When the females were selected as to stage of estrous, with metestrus including both pro- and post-estrus, it was found that there was no statistically significant difference in litter size between those in estrus and the controls, which latter group included at random probably all stages of estrous. Likewise, when those in diestrus and metestrus were compared with the controls, there was no statistical difference in litter sizes. However, when those in diestrus or metestrus were compared with those in estrus, it was found that there was a statistically significant difference at the 5% (or less) probability level (*i.e.*, $P \leq 0.05$). Nevertheless one could hardly categorize the increases as evidence of superovulation.

DISCUSSION

Superovulation is presumed to mean stimulation to excess ovulations in the normal ovary. Over many years the CF_1 mouse has been found to produce an average of 10.5 mature ova from both ovaries at each estrus, so that superovulation would presume a statistically significant increase in this number. Superovulation does not guarantee that the ova are entirely normal, nor that they could give rise to normal and viable offspring. To stimulate an ovary to excess or premature ova production would require some catabolic enzyme acting on the follicular membranes (Rugh, 1935) thus releasing extra ova. Also, there might be some stimulation to the maturation process from the anterior pituitary growth promoting hormone which might result in gross and precocious enlargement of each ovarian follicle. There are, of course, gonadotropic hormones from the mouse pituitary (as in all mammalian pituitaries) which stimulate maturation and release of ova (Rugh, 1935; Austin, 1950) which can then be fertilized. The anti-sterility, or fertility hormones now in use with the human patients do just that, but such ova are probably perfectly normal since they are released by hormones normal to ovulation in the human female. The point here is that mere superovulation is no guarantee of normality, except probably when appropriate hormones are used to accelerate the normal processes (*e.g.*, Amphibia).

It was not until 1954 and 1956 that the Russells first reported controlled experiments on female mice wherein X-radiation of the ovaries did temporarily seem to stimulate them to superovulate. Pozhidaev (1960) claimed that 600r to rats increased their corpora lutea by 50%, and since 1964 Hahn and coauthors have published fourteen papers (1964–1972), suggesting that ionizing radiations do indeed induce superovulation in their chosen strain of mice. Their most recent papers were in 1973 (Hahn and Feingold; Feingold and Hahn).

There remains some confusion about the matter—for rats, mice, hamsters, and humans do not react in precisely the same manner to ovarian X-radiation, and superovulation following X-radiation has not always been achieved. Further, some evidence suggests initial stimulation with subsequent sterility (Rugh and Wolff, 1956, 1957; Rugh, 1964). It was also shown by Muramatsu, Suguhara, Tsuchiya and Okozowa in 1965 that chronic low-dose exposure of female rodents during their reproductive period would result in at least three successive generations showing increased mean litter size, but that ultimately sterility would set in. Mondorf and Faber (1968) stated that human females X-irradiated in childhood were slightly more fertile than nonirradiated females, and Meyer, Merz and Diamond (1969) made a very extensive analysis of human data from an epidemiological study of pelvimetry to find that among black women, the birth rate was 10 to 15% higher among those who had been X-rayed *in utero* than among matched controls (Meyer and Tonascia, 1973).

Various explanations have been offered for the experimental and clinical findings, largely relating to hormonal imbalance, effects on the female genital tract (Hahn and Feingold, 1972), production of micronuclei in the ova (Harvey and Chang, 1963), hyperemia of the ovary, and the production of mutations. In some cases it seemed that the same explanation could be used for opposite effects, namely superovulation (stimulation) or sterility.

In a recent study (Rugh and Budd, 1975), it was shown that whole-body X-ray exposure of sexually mature female mice at any time from 10r to 500r prior to their mating with normal males of the same strain caused no significant change either in litter size or anomaly production. Ova exposed to even 500r, (which is about 120r short of the LD/50/30 dose for this strain of mice) were not killed and F_1 offspring from such ova were fertile and did not produce anomalous fetuses. There was slight reduction in litter size when the exposures were 300r and 500r, but the litters were found to be almost within the normal size range. These results were explained as being due to the protective effect of the normal genes from the nonirradiated normal sperm used to fertilize the irradiated ova, thereby masking any mutations induced in the ova. Mole (1959), Mandl and Zuckerman (1961), and Lacassagne, Duplan, Marcovich, and Raynaud (1962) had shown that whole-body X-radiation of female mice did result in impairment of fertility.

For the above reasons, emphasis in this current study is placed upon the numbers of offspring, rather than on numbers of corpora lutea and possible pre-implantation deaths.

CF_1 female mice go through an estrous cycle every 4.5 to 5 days, producing from 1 to 17 mature ova with an average of about 10.5. Since females selected at random could be in any phase of estrous, it was proper to allow one X-radiated cycle to pass through and be discarded so as to determine whether X-rays would stimulate the ovary to excess ova production among the oöcytes immediately ready for the succeeding cycle. To do this there was a delay of one week between X-radiation and mating (Table II). Changes in the average litter size were not significantly different, hence no induced superovulation. There is no basis for suggesting that an embryonic or fetal loss may have balanced out some presumed superovulation. Suggestions have been made that the age or stage of the oöcytes might make a difference in their responses to ionizing radiations. Thus the study was made on X-radiating the various stages of estrous (Womack and Bogart, 1969; Rugh, 1968). The study included 255 pregnancies and 2750 offspring; and the evidence suggested a slight reduction in litter size when the female was in estrus, but no superovulation at any time.

Whole-body X-radiation naturally includes the anterior pituitary gland, known as the master gland for reproduction, which might have an indirect effect on ovulation. Whether exclusive exposure of this gland might induce superovulation has not been reported. It is located in mid-center of the brain and would be difficult to find in focal X-radiation. Of course this gland is included in every whole body X-radiation, as are both ovaries (Mateykö and Edelmann, 1954). The knowledge that the anterior pituitary is the master reproductive gland led gynecologists and obstetricians to prescribe simultaneous pituitary and ovarian X-radiation of human females hoping to help them overcome their sterility (Kaplan 1938–1960; Fullenlove, Haman and Williams, 1956; Morgan and Reyes, 1957; Rugh and Clugston, 1954a, b). The fact that female mice of this strain tended to tolerate whole-body X-radiation better than did the males suggested to Rugh and Clugston (1954a, b) that there might be estrous cycle variations in response related to hydration changes in the phases of that cycle. This was found to be true, with diestrus being the most radiosensitive and estrus the least. This difference was attributed more to changes in body hydration than to hormone production. Rugh and Wolff (1956, 1957) found that all female mice, randomly selected with respect to estrus and exposed to whole-body X-radiation could all be sterilized by 20r within eight weeks of exposure, but that in the interim normal-sized and viable litters were produced. Thus, not only were the immediate stages of estrous shown to be relatively insensitive, but earlier stages in oöcyte production were sensitive enough to be killed by 20r exposures. These conclusions have been supported by Peters (1961) and by Mole and Papworth (1966), for mice and rats, but not shown for hamsters or the human.

It has been found by Brambell and Parkes (1927) that whether there was local ovarian or whole-body X-ray exposure of female mice there resulted the same effect on reproductive performance. But it was also noted, as long ago as 1931 by Murray, that the histological structure of the mouse ovary was not a good indicator of the mouse's age. Nevertheless an exposure of as little as 54r to the ovaries did cause structural changes of a histopathological nature, appearing as early as one day after exposure (Murray, 1931). It was also suggested that structural sterility occurred after 150r in two days. This latter observation is not confirmed, except possibly for the appearance of micronuclei in the hamsters which (Harvey and Chang, 1963; 1964) may not be proof of degeneration.

Harvey and Chang (1963) found in the hamster that the most radio-sensitive period in oöcyte maturation was eight to twelve hours *before* ovulation, during final enlargement of the follicle. They shielded the ovaries and adnexa on one side during X-radiation, and compared ova production from the ovary of the other side in the same animal. They found (1964) no increase or decrease in ova production, only a delay in the mitotic divisions and appearance of micronuclei which they considered evidence of cellular breakdown. Using up to 200r to hamster ovarian oöcytes during their first meiotic division, and mating with normal males three hours later, resulted in no change in litter size or production, and so few anomalies (8 out of 350 hamsters) that they were not considered significant. Harvey and Chang found that *corpora lutea were not increased by X-radiations* of the randomly selected hamster females to 200–700r. Mandl (1963, pp. 119, 135, 136) first suggested that X-radiation of the ovary on one side of a rat would reduce ovulations in the exposed ovary but cause compensatory increase in ovulations from the other and unexposed ovary of the pair, attributing this "as due to non-specific damage to the ovary mediated through a reduction in the number of pre-existing large Graäfian follicles undergoing atresia."

In 1972 Byskov and Peters found that when Bagg female mice were exposed to 300r whole-body X-rays and immediately mated with sexually mature males, that the resulting F_1 offspring showed early signs of aging and varied fertility but without change in the number of oöcytes maturing. However the F_2 mice appeared to produce more young than the controls, suggesting that any superovulating effect was carried through the germ cells for two generations. Hahn and Feingold (1972) found a transitory unilateral reduction of ovulations following unilateral ovarian X-radiation in the rat and Feingold and Hahn (1973) changed the initial word in the title of a report from "transitory" to "prolonged". In this latter instance they shielded the right lower quadrant of the female rat during X-radiation (400r) and recorded reduction of ova shed from the irradiated ovary and reproductive activity for 120 days stating that "the long term combined reproductive output of both sides (both ovaries) was not significantly different from contemporary unirradiated controls" (page 53).

Human ovaries and sometimes the pituitaries of the same individual have been exposed to ionizing radiations with the hope of overcoming sterility, with apparently some success even though the cause and effect relationship could not be irrefutably proven (Kaplan, 1938–1960). Gynecologists have contended that an ovarian wedge section is more likely to achieve ovulation in a human ovary than are X-rays. (Dr. H. Speert, College of Physicians & Surgeons, personal communication.) Some rat and hamster experiments have been reported in which a degree of excess ovulation has been described as relating to dose and time of irradiation during the estrous cycle (Hahn, *et al.*, 1964–1971). Our experiments indicate that in the species used, ionizing radiations did not stimulate excess or superovulation and consequently litter production. There has been no assessment of any possible genetic effects, ultimate sterility, or even possible teratogenesis, in future generations. It is virtually impossible to extrapolate these findings to the human, particularly with comparable dosimetry.

The author expresses his appreciation for the statistical analysis made by Dr. Gordon L. Jessup, Jr.

SUMMARY

1. The average litter size for normal CF_1 mice is close to 10.5, varying in these experiments from 9.75 to 11.73. Separate groups of controls were provided for each of the three major experiments.

2. Resorptions were found even among the control groups, with an average of 1.5%. Other anomalies were not encountered, even in offspring from irradiated ova. Resorptions following X-radiation of ovarian ova never exceeded 7%, which is still within the normal range.

3. Depending upon the experiments, sexually mature females were either taken at random with regard to their estrous cycle, or vaginal smears were made and the females categorized to select the particular phase of estrous cycle of concern.

4. Among litters from matings immediately after (whole-body) X-radiation from 10 to 200r, the variations in resorptions over exposure levels was minimal, while variation in average litter size was within the range of normal experience for these mice.

5. Even among mice mated one week after exposure the differences in litter sizes were small and the statistical significance tenuous.

6. It was found that even a maximum exposure of 200r altered neither the resorption rate or production of anomalies, nor induced any significant increase in litter size.

7. When the various phases of the estrous cycle were segregated for irradiation to 100r, it was found that those in estrus showed somewhat smaller mean litter size than those which were X-irradiated during diestrus or metestrus. However, when compared with the control group, which probably included all phases of the estrous cycle, there was no statistically significant change.

8. On the basis of this study of some 8000 offspring, there is no evidence of significant change in ova production following whole-body X-radiation from 10r to 200r of mature CF_1 female mice, and certainly no evidence of superovulation.

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LEAD UPTAKE IN TWO MARINE PHYTOPLANKTON ORGANISMS

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Hundreds of thousands of tons of lead are discharged annually into the earth's atmosphere in the exhaust gases of internal combustion engines fueled with leaded petroleum, *i.e.*, hydrocarbon mixtures supplemented with the antiknock agent lead tetraethyl. From the atmosphere the metal, largely as oxides and salts, is washed down by rain to the surface of the earth. The surface layer of the ocean, the euphotic zone, is thus particularly susceptible to lead input. The possibly hazardous effects of this element on phytoplankton have been studied for only a few species, mostly hardy laboratory organisms; they indicated a rather high tolerance to lead (Hessler, 1974; Malanchuk and Gruendling, 1973). However, even if lead at present environmental levels in the ocean is not appreciably toxic, its uptake by the phytoplankton, at the first level of the marine food chain, is of great ecological importance, since such organisms may not only play a role in the geochemical distribution of lead, but also, by serving as food, transport the lead to higher trophic levels. For example, lead-enriched algae can increase the lead content of a bivalve mollusc, *Mytilus edulis*, to the same extent as elevated lead concentrations in the surrounding medium (Schulz-Baldes, 1974).

In this paper we indicate the high potential of certain algal cells to take up lead, supplied at sub-toxic levels, quickly and completely from the medium.

Materials and Methods

Algal cultures

Axenic cultures of the diatom *Phaeodactylum tricornutum* (originally isolated by R. A. Lewin in Woods Hole, Massachusetts) and the green flagellate *Platymonas subcordiformis* (originally isolated by R. A. Lewin from Morro Bay, California) were grown in Erlenmeyer vessels at a constant temperature of 20° C. The cultures were continuously shaken on a reciprocating shaker (120 oscillations/min) and illuminated by "cool-white" fluorescent tubes (2000 lux).

The culture medium for *Phaeodactylum* consisted of filtered local sea water with additions of potassium nitrate (1 g/l), sodium glycerophosphate (0.1 g/l), and a trace-mineral solution containing Fe, etc. (Lewin and Lewin, 1967). The culture medium for *Platymonas* consisted of filtered local sea water with additions of sodium glutamate (1.7 g/l), sodium glycerophosphate (0.1 g/l) and the trace-mineral solution. For experiments on the uptake of lead, cells in the exponential phase were centrifuged from the enriched medium and resuspended in fresh, unenriched sea water which had been filtered through a 0.45 μm Millipore filter and then autoclaved. In this way, we attempted to minimize chelation of lead ions by the EDTA or glutamate present in the culture medium, to reduce interference by other cations, and to retard cell multiplication.

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Lead was administered from a stock solution containing 1 g Pb/l (as chloride, in 0.01 N HCl). After treatment with known concentrations of lead, some cell samples were extracted with the chelating agent EDTA (prepared as a stock solution containing 0.2 M) to determine how much of the adsorbed and/or absorbed Pb could be readily eluted. After EDTA treatment, in most experiments for one hour, the cells and the medium were analyzed in the same way as the cultures described below. During the experimental periods of 5–7 days the cell numbers, routinely determined with a Coulter Counter, increased by only about 30%.

Lead determinations

The contents of lead in the algae and the medium were determined by flameless atomic-absorption spectrophotometry, using a Perkin-Elmer model 403 with a heated graphite tube HGA-2000 and a deuterium background corrector. The algae were centrifuged from the medium, suspended in 0.1 N HCl, counted, transferred to a smooth graphite tube, and assayed for their lead content by the method of standard additions. The determination of lead in the medium was carried out after an extraction step using a solution of 2% ammonium pyrrolidine dithiocarbamate (APDC) and methyl isobutyl ketone (MIBK), according to a modification of the micro-method of Kremling and Petersen (1974). The MIBK extract was measured in a grooved graphite tube.

In this paper the lead associated with the algae ("particulate" lead) is generally given as mg lead bound to the algal cells contained in one liter of medium, allowance being made for cell growth and biomass increase during the experiment. This value can then be directly compared with the lead concentration in the medium (soluble lead). However, in Figure 4 the lead content is expressed as μg per gram dry weight.

Dry weights per cell were estimated to be 2×10^{-11} g for *Phaeodactylum* and 6×10^{-11} g for *Platymonas*.

RESULTS

The course of lead uptake by *Platymonas* and *Phaeodactylum* in batch assays exhibits two phases. In both species the first phase is a rapid increase in the lead associated with the algae. Although the first measurement in the experiments illustrated in Figures 1 and 2 was not carried out until one hour after the addition of lead, other experiments showed that the first phase takes only a few minutes. The second phase shows a pattern which differs for the two algal species. The lead content of *Platymonas,* after a slight decrease in the first day, steadily increases during the next 7 days, whereas after day 1 the lead concentration in the medium remains very low (Table I). The lead content of *Phaeodactylum* in the second phase increases until the second day, but then declines, and after day 2 there is a corresponding increase in the concentration of lead in the medium (Table II).

The sum of the measured amounts of soluble and "particulate" lead was generally less than the total amount of lead originally added to the medium: *e.g.*, in media initially containing 0.3 mg Pb/l, some 20% to 40% remained unaccounted for (see Fig. 3). The "missing" fraction had probably been adsorbed to the surface of the experimental vessel, from which it could later be desorbed, and subsequently taken up by the algae, as the concentration of soluble lead decreased.

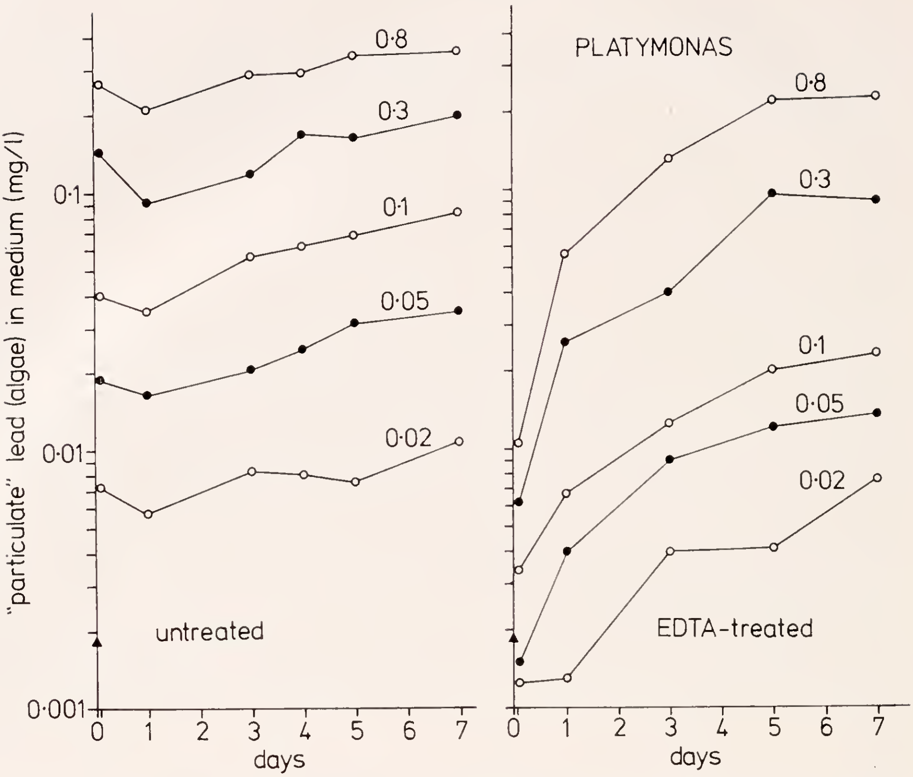

FIGURE 1. *Platymonas subcordiformis*: lead content (= "particulate" lead, expressed as mg Pb bound to the algal cells contained in one liter of medium) of cells suspended in a medium containing Pb (0.02–0.8 mg/l) and then untreated (left) or treated with 10^{-2} M EDTA for one hour (right), as a function of time. In all figures, the black triangles indicate the natural lead content of the cells. Cell numbers per ml rose from 3.2×10^6 (day 0) to 4.5×10^6 (day 7).

The uptake during the first phase can be described by a Freundlich adsorption isotherm $x/m = k \times c^n$, where x is the amount adsorbed, m, the mass of adsorbent, c, the concentration in solution, and k and n, the constants. The first equation can be rearranged to give a straight line in a log-log plot: $\log x/m = \log k + (n \times \log c)$.

TABLE I

Pb concentration determined in medium, Platymonas subcordiformis, *cultures.*

Day	Hour	Lead initially added to medium (μg/l)				
0	0	20	50	100	300	800
0	1	5	6	9	48	143
1		<4	<4	6	36	63
3		<4	<4	<4	21	32
5		5	<4	7	18	38
7		<4	<4	5	16	30

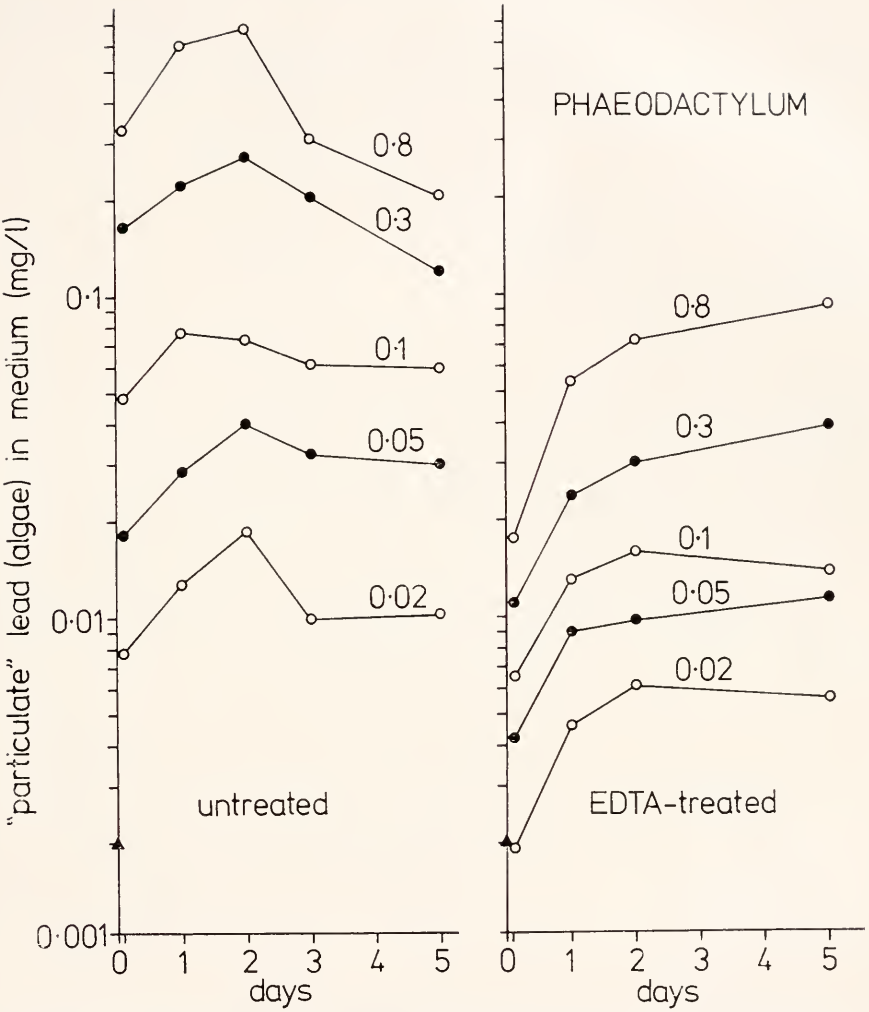

Figure 2. *Phaeodactylum tricornutum*: lead content of cells, as a function of time; see legend to Figure 1. Cell numbers per ml rose from 7×10^6 (day 0) to 10×10^6 (day 5).

Table II

Pb concentration determined in medium, Phaeodactylum tricornutum, *cultures.*

Day	Hour	Lead initially added to medium (µg/l)				
0	0	20	50	100	300	800
0	1	<4	6	20	47	194
1		<4	<4	7	18	85
2		<4	7	13	18	90
5		7	14	35	118	382

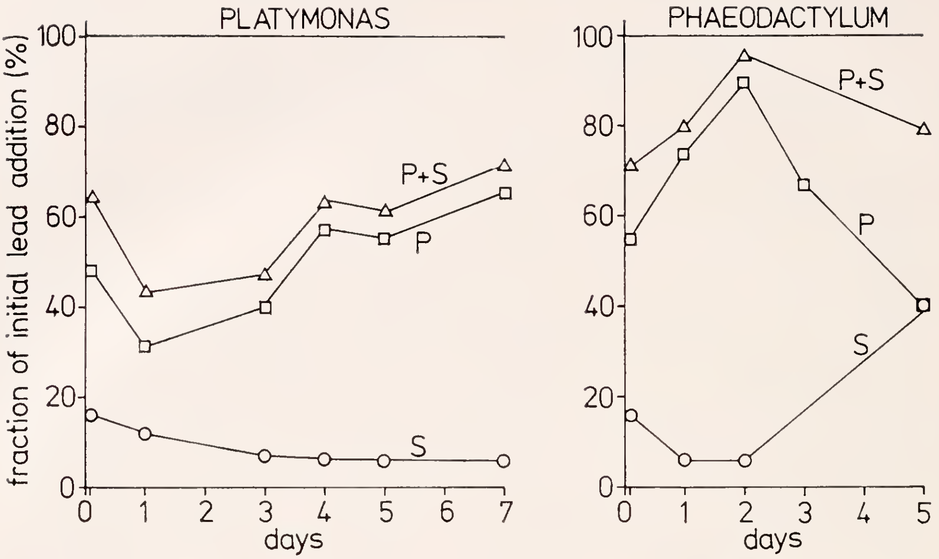

FIGURE 3. *Platymonas subcordiformis* and *Phaeodactylum tricornutum*: lead budget in percent of initial addition (0.3 mg Pb/l) as a function of time. S indicates soluble lead; P, "particulate" lead, *i.e.* lead associated with the algae; and P + S, sum of soluble and "particulate" lead.

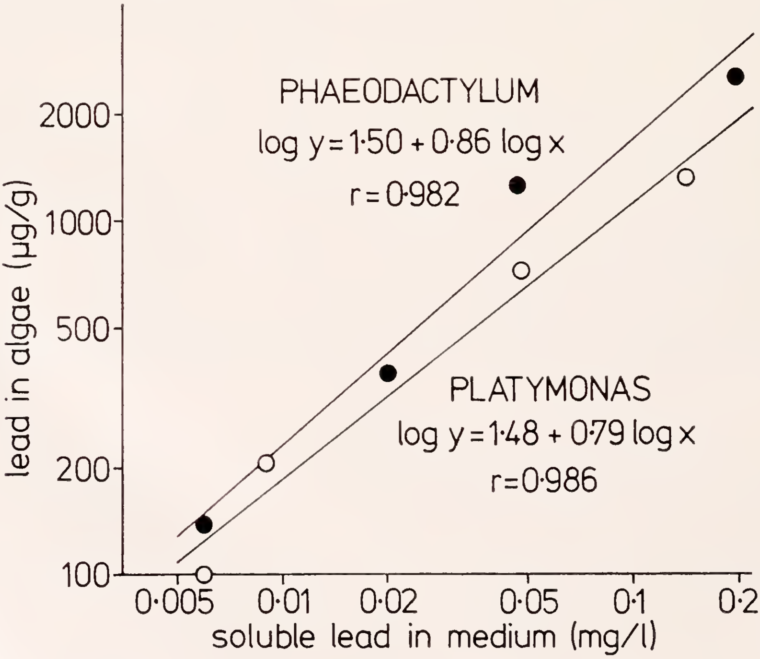

FIGURE 4. *Platymonas subcordiformis* and *Phaeodactylum tricornutum*: Pb adsorption isotherms after one hour of lead exposure.

TABLE III

Cells of Phaeodactylum tricornutum *"saturated" at the surface with lead, measured 10 min after lead addition.*

Cell number (10^9 cells/l)	Pb addition to medium (mg/l)	Pb adsorbed to cells	
		(mg/l)	(μg/g dry weight)
0.63	1	0.154	11,860
1.25	2	0.282	11,280
2.5	4	0.589	11,780
			Average: 11,640

Figure 4 shows the adsorption isotherms for *Phaeodactylum* and *Platymonas* after one hour of lead exposure. [The values for the lowest lead addition (0.02 mg/l) have not been used for this figure as the very low concentration in the medium (<0.004 mg/l) could not be determined exactly]. The data for both algae are highly correlated with the lines fitted by the method of least squares. The resulting logarithmic equations can be converted to the following exponential equations: *Phaeodactylum*, $y = 31.66\,x^{0.86}$; and *Platymonas*, $y = 30.25\,x^{0.79}$.

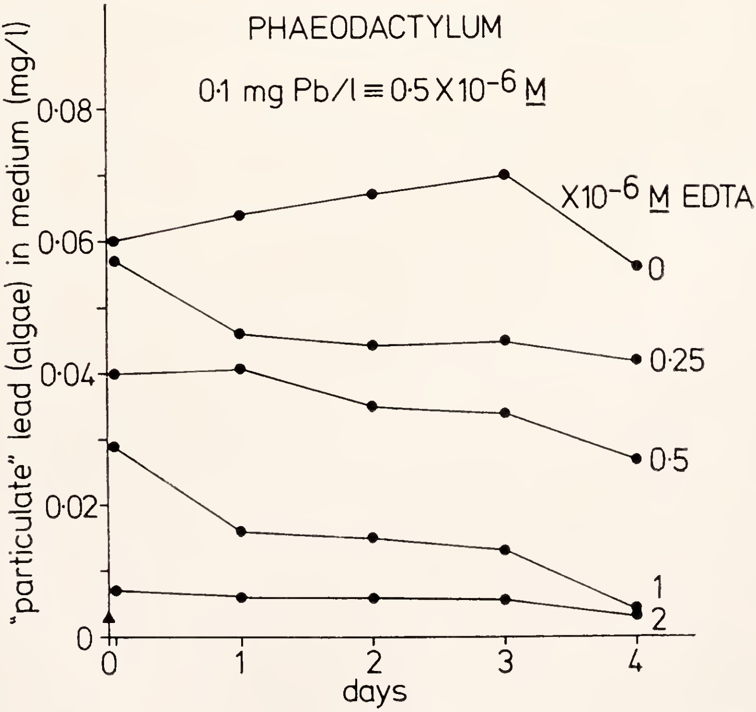

FIGURE 5. *Phaeodactylum tricornutum*: lead content of cells exposed to 0.1 mg Pb/l in the presence of 0 to 2×10^{-6} M EDTA, as a function of time.

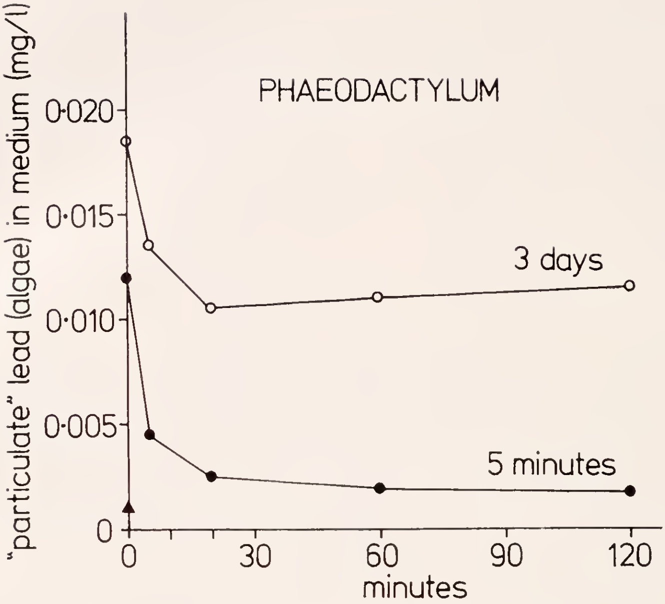

FIGURE 6. *Phaeodactylum tricornutum*: lead content of cells, previously exposed to 0.02 mg Pb/l for 5 min or 3 days, and then treated with 10^{-2} M EDTA, as a function of time.

The capacity of *Phaeodactylum* cell surfaces to adsorb lead was determined by supending cells for ten minutes in media containing a rather high concentration of dissolved lead, removing them by centrifugation, and determining the amounts of lead taken up. Despite differences in cell numbers and lead concentrations, we found that the same amount of lead was adsorbed per cell (Table III), suggesting a limited number of binding sites. These were all occupied by a lead load of 11,640 μg/g dry weight, equivalent to 2.3×10^{-13} g or 6.7×10^{8} Pb-atoms per cell. Considering the surface of a *Phaeodactylum* cell as a frustum of a right double cone 20 μm long and 6 to 3 μm wide, with an area of 440 μm^2, we calculate that about 1.5 atoms of lead can be absorbed per nm^2.

Only uncomplexed lead is available for adsorption, as demonstrated in an experiment with different concentrations of EDTA added immediately before the lead treatment. About 2×10^{-6} M EDTA completely inhibits lead uptake from a 0.5×10^{-6} M solution (0.1 mg Pb/l) (Fig. 5). Probably other ions are chelated, too, but even at lower EDTA concentrations some of the lead is eluted from the algae after its initial adsorption. In order to test how tightly the lead is bound on the algae, "leaded" cells were treated with 10^{-2} M EDTA. This high concentration (which kills the algae within 24 hours) within 20 minutes reduces the lead content of the cells to a certain level which is evidently dependent on the previous time of lead exposure (Fig. 6). The bound lead content of *Platymonas* and *Phaeodactylum*, *i.e.*, that fraction which remains associated with the cells after a one-hour treatment with 10^{-2} M EDTA, increases considerably with time (Figs. 1 and 2). In EDTA-washed cells of *Phaeodactylum* the lead content seems to be more or less constant after day 2. By contrast, in *Platymonas* the lead loss after EDTA treatment is initially slightly higher than in *Phaeodactylum*, but later there is a distinct increase in bound lead which, after about seven days, seems to reach an equilibrium at a level much higher than in *Phaeodactylum*.

DISCUSSION

When a lead solution is added to sea water the metal becomes distributed between the medium, the vessel surface, and (if enough is added) precipitates. At concentrations higher than 1 mg/l in sea water, lead tends to be precipitated as carbonate and phosphate. There is no loss by evaporation, as in the case of mercury (Davies, 1974). However, it is not known precisely in what chemical species the lead occurs in such solutions. Zirino and Yamamoto (1972) reported that in sea water there could be a variety of complexes: $PbCO_3^0$, $PbCl^+$, $PbCl_2^0$, Pb^{2+}, $PbCl_3^-$ and perhaps $PbOH^+$. Complexing and chelation by organic ligands in natural sea water are also to be expected, as pointed out in the case of copper by Davey, Morgan and Erickson (1973). In algal cultures, some of the lead is adsorbed or absorbed by the cells, and the fraction of chelated lead can be expected to increase if the cells liberate anionic colloids as extracellular metabolites. In fact, Hellebust (1965) reported that *Phaeodactylum* secretes up to 7% of the total assimilated carbon, some of it as polysaccharides liberated from cell surfaces, which could act as binding agents for lead.

The initial rapid increase in the lead content of the cells seems to be due almost entirely to the physicochemical process of adsorption to the cell surface. Afterwards the metal may proceed by active transport or by diffusion to other sites. Whether there is a true uptake of lead into the cytoplasm, or whether the lead is only bound to the cell wall or the plasma membrane, may have to be confirmed by microchemical methods similar to those employed by Ophus and Gulvåg (1974) in their studies of lead uptake by the moss *Rhytidiadelphus squarrosus*. By electron microscopy in connection with X-ray microanalysis, they demonstrated the presence of lead and phosphorus within nuclear and chloroplast inclusions. The variation in the lead content of EDTA-treated cells also seems to indicate a change in the location of the bound lead during extended periods of exposure to solutions containing the metal. In both algal species there is a considerable increase, with time, in the bound lead, *i.e.*, that fraction which is not removable by EDTA, suggesting that the metal is moving, or being translocated, into the cells. In a similar way, Davies (1970, 1973) used this method to distinguish between iron or zinc absorbed on the exterior surface, and intracellular metal ions.

In another respect our results exhibit a striking similarity to those obtained by Davies (1973) on zinc uptake in *Phaeodactylum*. Loss of zinc from the cells begins about 19 hours after zinc addition, leading Davies to postulate a reduction in the number of zinc-binding sites (probably protein) within the cells as they go through their growth cycle. We, too, noted such a decline in lead content, although in our experiments the EDTA-treated cells show no loss of lead after the second day (Fig. 2). (Possibly the released lead is not readsorbed because it is complexed or chelated by organic material in the medium. The method we employed for the determination of lead in the medium involves the use of a very strong chelating agent, and thus does not distinguish between more weakly chelated and ionic lead.)

Measurements of lead bound on the algae and lead in the medium never added up to account for all of the lead originally added: in most cases only 60% to 80% could be found. One possible explanation for the observed "loss" of Pb was its adsorption on the walls of the glass vessels. To test this we assayed the dissolved lead in an Erlenmeyer flask containing filtered and autoclaved sea water to which lead (0.1 mg/l) had been added and which was continuously shaken and illuminated. After 7 days the lead concentration in solution had decreased by 23% probably due to adsorption on the glass. The adsorption characteristics of lead on borosilicate glass have been described in detail by Struempler (1973). Presumably when algal suspensions are assayed some of the cells and other organic matter also stick to glass, thus further enlarging the adsorptive surface and scavenging more lead. On the other hand, some of the previously adsorbed lead might be later released from the vessel surface during depletion of lead in the medium by algal activity, and then taken up by the cells. This may explain the increase in the lead content of the algae while the concentration of dissolved lead in the medium was at a very low level.

In spite of this somewhat unsatisfactory balancing of the lead budget in our experiments, we think we have demonstrated unequivocally that lead ions first are quickly and reversibly bound to the cell surfaces, and only later penetrate to deeper sites (from which they can be less readily dislodged with EDTA) where they may be expected to exert their main biological effects.

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Summary

1. Uptake of lead by *Phaeodactylum tricornutum or Platymonas subcordiformis,* exposed to lead concentrations ranging from 0.02 to 0.8 mg/l, occurs in two phases.

2. The first phase, completed within minutes after addition of lead, can be described by a Freundlich adsorption isotherm. The number of binding sites per cell seems to be limited. Cells of *Phaeodactylum* become "saturated" when the lead burden reaches 11,640 μg/g (dry weight), equivalent to about 6.7×10^8 Pb atoms per cell.

3. In the second phase, the lead content of *Platymonas* cells continues to rise slowly, whereas that of *Phaeodactylum* declines after two or three days.

4. The addition of 2×10^{-6} M EDTA to a solution containing 0.5×10^{-6} M Pb completely inhibits the uptake of the metal by *Phaeodactylum* cells. When diatom cells, pre-treated with lead, are resuspended in a higher concentration of EDTA, 10^{-2} M, much of the adsorbed lead is eluted. The longer the pre-treatment period with lead, the less readily is the metal removed from the cells in this way.

5. Since in both species the content of bound lead, *i.e.*, the residual lead burden after EDTA extraction, increases with time, we suggest that during prolonged exposure to lead solutions the metal ions are first adsorbed to the cell surface and then translocated to within the cell wall, to the plasma membrane, and eventually to the cytoplasm.

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THE TAXONOMY, DEVELOPMENT AND BROODING BEHAVIOR OF THE ANEMONE, *CRIBRINOPSIS FERNALDI* SP. NOV.

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The Actiniidae is the largest and most diverse of the families of sea anemones (Anthozoa: Actiniaria). In addition to the morphological diversity within this family, there is considerable diversity in larval development. The Actiniidae are generally considered to be highly advanced anemones, although Hand (1966) has argued that this family should be considered primitive.

In this paper we present information on the taxonomy and life history of the anemone *Cribrinopsis fernaldi* sp. nov. The brooding behavior exhibited by this anemone is intermediate between that of the nonbrooding anemones such as *Metridium dianthus* and *Adamsia palliata* (Gemmill, 1920) and the specialized brooding anemones such as *Actinia equina* (Chia and Rostron, 1970).

MATERIALS AND METHODS

Adult anemones were collected at various subtidal areas near San Juan Island, Washington using S.C.U.B.A. The anemones were maintained in sea water tanks at the Friday Harbor Laboratories.

The following taxonomic description is based on the study of thirty living or preserved specimens and follows the pattern of Hand (1954, 1955). The cnidom was determined according to the method of Hand (1954) and the descriptions of the nematocysts follow the system of Weill (1934), as modified by Carlgren (1940a). A minimum of 50 nematocysts of each type was measured. Several specimens were dissected to determine the arrangement of mesenteries and distribution of gonads and serial longitudinal and cross sections were prepared from four specimens. After relaxation in 7.5% magnesium chloride, the anemones were fixed in Bouin's fluid or 4% formalin-sea water, embedded in paraffin, sectioned at 15 μm, and subsequently stained with basic fuchsin followed by picro-indigocarmine.

Four anemones collected during the last week of March, 1971 provided the material for the developmental study. Two female and one male anemone (sex determined by postspawning analysis) were placed together in a seawater tank. A single female was placed in an adjacent tank. The male was observed releasing sperm on March 30. At the same time eggs could be seen in the gastrovascular cavity of the females. The eggs, embryos and larvae were removed from the gastrovascular cavity of the females by clipping the end from a tentacle and applying gentle suction with a basting syringe. Embryos obtained in this manner were either prepared immediately for histological observations or cultured. Cultures were started on days 1, 7, 13, 34, and 40 of development.

Embryos and larvae were cultured in jars filled with 500 ml of sea water. The cultures were maintained at approximately 12° C and the water changed every second day. After the larvae settled they were fed *Artemia* nauplii.

Histological preparations were made simultaneously of individuals removed from the adult females and those removed from the cultures. They were fixed in Bouin's fluid and embedded in paraffin. Serial sections were cut at 5–8 μm thickness and stained with Heidenhain's hematoxylin and Orange G.

TAXONOMY

Family Actinidae, Gosse, 1858. The definition of Carlgren (1949) remains unchanged.

Genus *Cribrinopsis*, Carlgren, 1921. The description of Carlgren (1949) should be amended to read as follows (Italics indicate amendments): Actiniidae with usually feebly developed verrucae on the column *or verrucae absent.* Pedal disc is well developed; pseudospherules are present or absent; fosse distinct. The sphincter is strong, palmate or pinnate circumscribed. Tentacles are simple, sometimes papillose, short, thick, or *long, tapering, and more or less thickened.* Longitudinal muscles of the tentacles are principally mesogleal, radial muscles or oral disc are meso-ectodermal to ecto-mesogleal. Numerous perfect mesenteries are decamerously, hexamerously, or irregularly arranged; well developed mesenterial muscles. Gonads are on mesenteries of the first cycle and on the other stronger mesenteries, often absent on the directives. Mesenteries are more numerous proximally than distally. The basitrichs of tentacles and actinopharynx are about the same length. Cnidom: spirocysts, basitrichs, microbasic *p*-mastigophores, *atrichs.*

Cribrinopsis fernaldi sp. nov., Siebert and Spaulding.

Description

The base is circular, often irregular in outline, adherent and often transparent. The mesenterial insertions may be visible or not. The basilar muscles are strong.

The column (Figs. 3b, c) is smooth, without adherent material and the verrucae are very weak or absent. Small white tubercles arranged in numerous longitudinal rows are present and the column is usually as high as wide, or higher. The collar and fosse are well developed and numerous marginal pseudospherules are present. The color of the column is variable, generally white, pink, or yellow and as the tissue of the column is very thin, it sometimes appears translucent or transparent with the mesenteries visible along the inner face of the column. The verrucae and tubercles generally are restricted to the upper one third of the column, but sometimes extend almost to the limbus. Cinclides and pores are absent.

The tentacles (Figs. 3b, d) are long, tapering from base to tip with cinclides at the tip and are usually white in color with thin irregular pink or yellow stripes encircling them. The ectoderm is smooth, thin and never papillose. The arrangement is hexamerous, in up to five cycles (96 tentacles). The number may vary somewhat and individuals with 92 to 104 tentacles are not uncommon. The longitudinal muscles of tentacles are well developed, ectomesogleal.

The disc is the same color as the tentacles and the column, often semi-transparent and with mesenterial insertions visible. There may be thin red or yellow lines around the bases of the tentacles, sometimes extending from the margin to the lips.

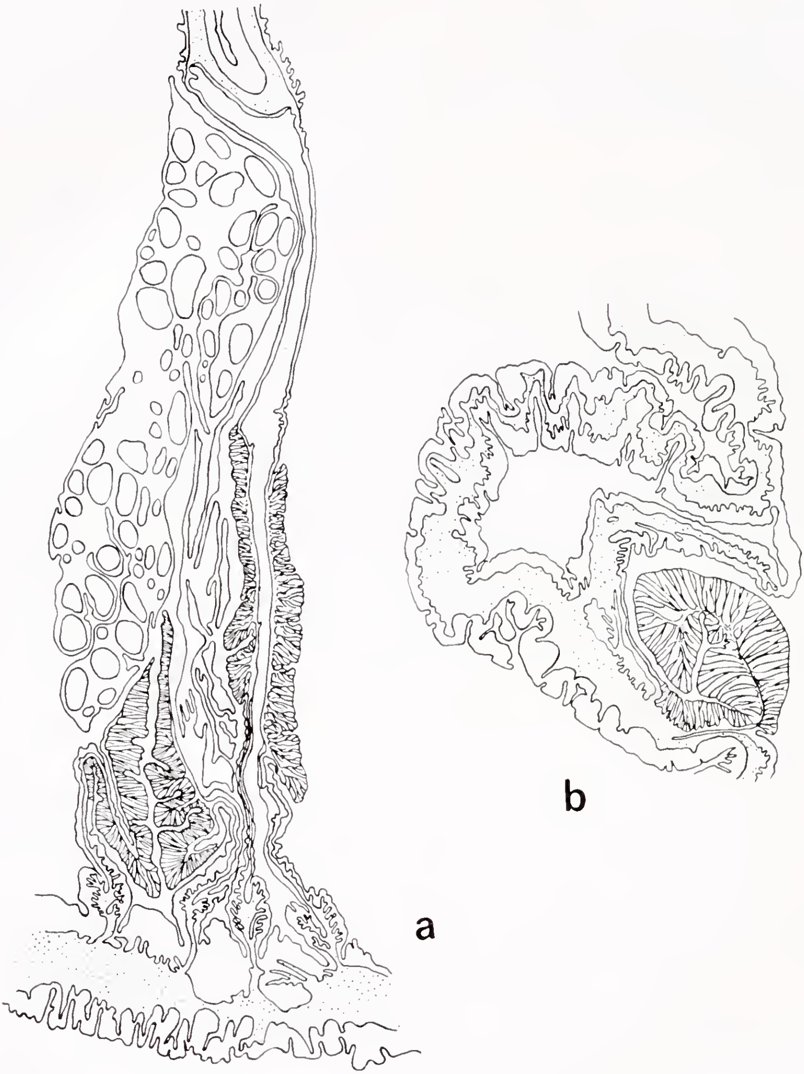

FIGURE 1. a) Cross section of directive and fifth-order mesenteries, 11×; b) longitudinal section of column and oral sphincter, 12×.

The disc is broad and flat, with the lips usually raised and folded and they are light pink in color. The actinopharynx is thick, glandular, and heavily ribbed and two thick-walled siphonoglyphs are present. The radial muscles of the disc are well developed and mesoectodermal.

In fully developed specimens 96 pairs of mesenteries are present, hexamerously arranged, through variations in this number my occur in some specimens. Gonads are present on all mesenteries except the two directive pairs. The retractors are large, strong and diffuse to somewhat restricted (Fig. 1a) and the laminae of the

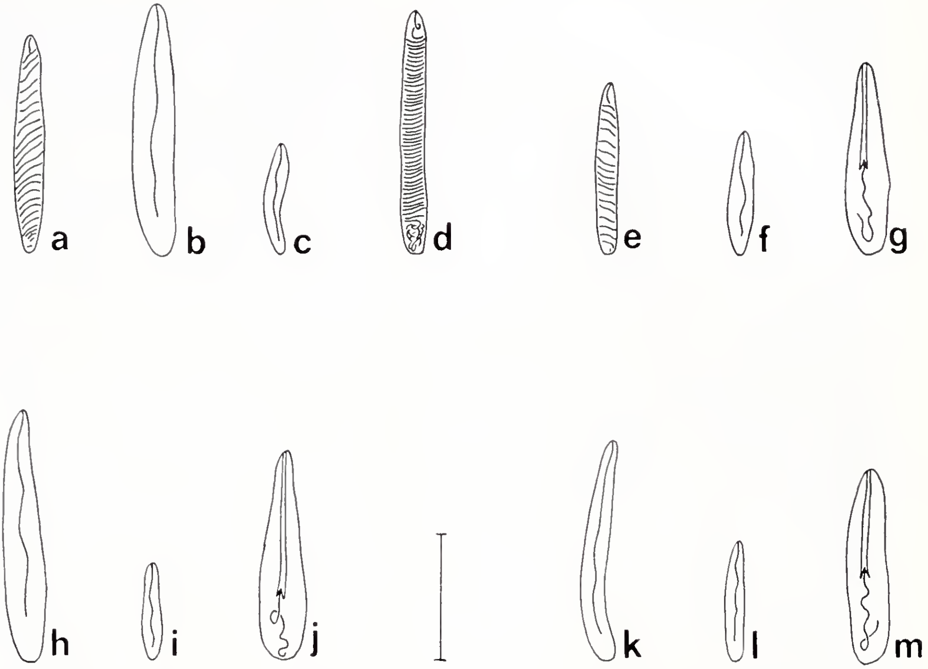

FIGURE 2. Nematocysts of *Cribrinopsis fernaldi* (all nematocysts are drawn to same scale, bar equals 20 μm). a-d) Tentacles; e-g) column; h-j) actinopharynx; k-m) filaments; (a and e) spirocysts, (b, c, f, h, i, k, and l) basitrichs, (d) atrich, (g, j, and m) microbasic *p*-mastigophores.

muscles are thin and finely branched. The parietal and parietobasilar muscles are well developed.

The sphincter (Fig. 1b) is strong, pinnate circumscribed and attached to the column just below the fosse. The laminae are of unequal length throughout the sphincter and branched.

The cnidom (Fig. 2) consists of spirocysts, basitriches, microbasic *p*-mastigophores and atrichs.

The distribution and size of the nematocysts (all measurements in microns) is as follows:

Tentacles	
Spirocysts	16–55 × 2.0–4.5
Basitrichs	28–67 × 2.5–5.0
Basitrichs	12–23 × 2.0–3.0
Atrichs (Rare)	27–51 × 3.0–4.0
Column	
Spirocysts	15–39 × 1.5–3.5
Basitrichs	15–26 × 2.0–3.0
Microbasic *p*-mastigophores	23–37 × 4.0–7.0

Pharynx

Basitrichs	26–59 × 3.0–6.0
Basitrichs	12–18 × 2.0–3.0
Microbasic *p*-mastigophores	29–38 × 4.5–7.0

Filaments

Basitrichs	29–42 × 5.0–6.0
Basitrichs	12–30 × 2.0–3.5
Microbasic *p*-mastigophores	26–40 × 4.5–7.0

The size of an average individual may be 5–8 cm in diameter at the base, 6–9 cm tall, and 6–8 cm in diameter across the disc. The tentacles in a specimen of these dimensions would be 6–8 cm in length.

This anemone is found on rock walls sloping to vertical, which are heavily encrusted with coralline algae, *Allopora* (a stylasterine hydrozoan), bryozoans, and sponges at a depth of 25–35 meters. Other larger invertebrates common in the same area include *Metridium senile, Terabratalia transversa, Cucumaria miniata, Psolus chitinoides, Evasterias troschellii,* and *Chlamys* spp.

The type locality is about ten meters northeast of Cantilever pier, University of Washington, Friday Harbor Laboratories, San Juan Island, Washington.

The type specimens were deposited in the U. S. National Museum. The holotype is USNM No. 54132; and the paratype is USNM No. 54133.

Diagnosis and remarks

This anemone may be distinguished by the long, tapering tentacles which are usually white with irregular red or yellow stripes, the presence of marginal pseudospherules, and the rows of white spots along the column.

The genus *Cribrinopsis* was established by Carlgren (1921) and at that time he described *C. similis* from the North Atlantic as the type for the genus. Later, he (Carlgren, 1940b) described *C. williamsi* from the Northeast Pacific. Parulikar (1966) records an undescribed species of *Cribrinopsis* from the Indian coast, but gives scant information concerning it, and Schmidt (1972) added *C. crassa* (Andres, 1884). *Cribrinopsis fernaldi* differs significantly from each of the previously described species of the genus in several respects.

The three previously described species of *Cribrinopsis* all have thick tentacles which may be papillose while those of *C. fernaldi* are thin and tapering. There are 60–90 tentacles in *C. similis,* approximately 48 in *C. williamsi,* and 96 in *C. crassa. Cribrinopsis fernaldi* generally possesses 96. All species have verrucae on the column, though in *C. fernaldi* they may be so weak as to be absent. *Cribrinopsis similis* lacks marginal pseudospherules while the other three described species possess them. In *C. similis* the mesenteries are decamerously arranged, those in *C. williamsi* may be decamerous or hexamerous, and in *C. crassa* hexamerous. In *C. fernaldi* the mesenteries are hexamerous.

From the above comparison, it can be determined that *C. fernaldi* is similar to *C. crassa* in the number and arrangement of the tentacles and mesenteries and distinct from *C. similis* and *C. williamsi.* However, *C. fernaldi* and *C. crassa* differ greatly in the shape and patterns of the bands of color on the tentacles and in the

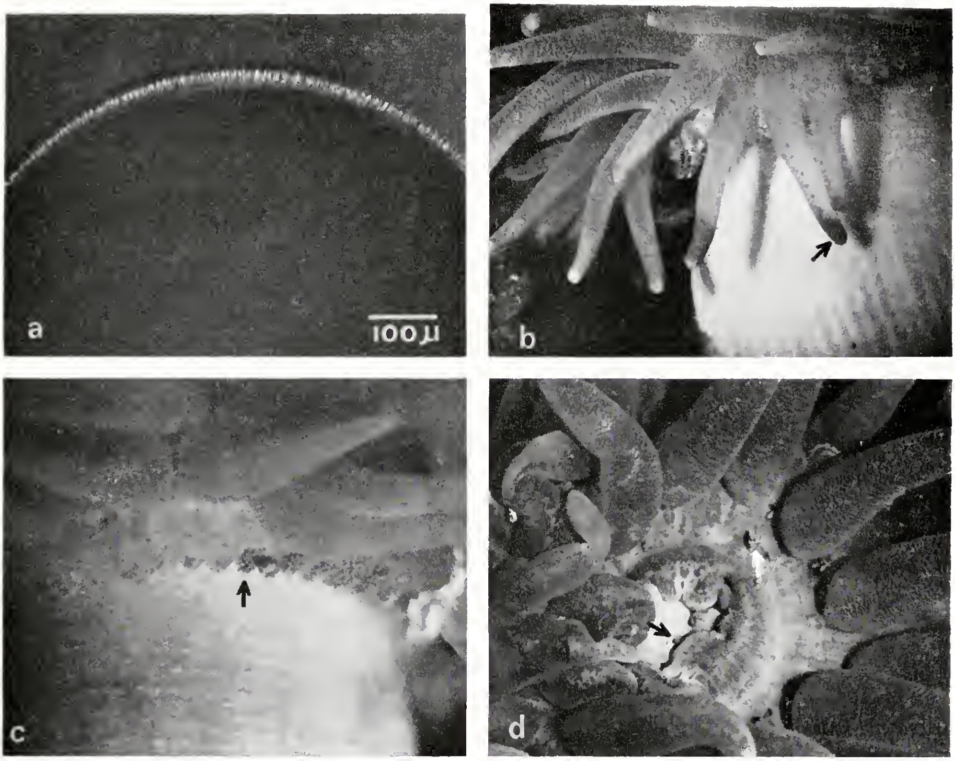

FIGURE 3. a) Recently spawned egg. Spiny surface is emphasized by phase contrast; b), an adult female with fertilized eggs in the tentacles and pseudospherules (arrows); c), a closer view of the eggs in pseudospherules (arrow); d), the same female releasing planulae (arrows).

size and shape of the verrucae. In *C. crassa* these are quite large and distinct while in *C. fernaldi* the verrucae are small and inconspicuous, if not absent. The ectoderm and mesoglea of the column, disc, and base is much thicker and better developed in *C. crassa* than in *C. fernaldi*. In addition, the cnidom of each species is distinctive.

Available data concerning the cnidom of *C. crassa* is not complete, though there is enough information available so that a meaningful comparison of the cnidoms of *C. crassa* and *C. fernaldi* is possible. The data of Schmidt (1972) are summarized below.

Distribution and size of nematocysts of *C. crassa* (all measurements in microns).

Tentacles	
Spirocysts	21–39 × 2.5–4.0
Basitrichs	21–32 × 2.0–3.0
Pharynx	
Basitrichs	26–38 × 2.5–4.5

Filaments

Basitrichs	30–46 × 3.0–5.5
Basitrichs	13–20 × 1.5–2.5
Microbasic *p*-mastigophores	21–28 × 3.5–5.5

The distribution of nematocysts appears to be somewhat different in the two species and the sizes of the nematocysts are also very dissimilar. This is particularly noticable when comparing the large basitrichs of the tentacles and pharynx of both species. The largest basitrich recorded from the tentacles of *C. crassa* is 39 μm and for *C. fernaldi* 55 μm, while the largest basitrich from the pharynx of *C. crassa* is 38 μm and for *C. fernaldi* 59 μm. A further comparison of the cnidoms of these species will reveal additional differences.

Though atrichs are common in various genera in the family Actiniidae, such as *Anthopleura* and *Actinia,* they were previously unrecorded from the genus *Cribrinopsis.* As these were located only on the tentacles and are rather rare, it is not surprising that they have been overlooked until now. No great significance can be attached to their presence in *C. fernaldi* until further studies are made of the cnidoms of the other species in the genus.

DEVELOPMENT

Spawning

C. fernaldi is dioecious. The male was observed releasing sperm from its mouth late in the morning of March 30. These sperm were being taken into the gastrovascular cavities of the females in currents of water. Eggs could be seen free of the gonads inside the gastrovascular cavities of all three females. These eggs were primarily in the tentacles and pseudospherules (Figs. 3b, c).

The eggs are 700–750 μm in diameter and cream to pale pink in color. Observations of the living eggs using phase contrast microscopy revealed that the oölemma is formed into conical spines about 15 μm in length (Fig. 3a). Histological examination of sectioned material indicated that each spine visible in a living egg was composed of a group of cytospines (Dewel & Clark 1974).

Development

Comparison of developing individuals removed from the females at various times and cultured prior to fixation and those fixed immediately upon removal showed no difference in morphology or rate of development. The following description applies to both the brooded and cultured organisms. A summary of the development and experimental observations is given in Table I.

The eggs in the gastrovascular cavity of the two females in the tank with the male began to show cleavage by late afternoon. Those in the isolated female did not develop, but remained in the gastrovascular cavity. Cleavage is preceded by a few rounds of nuclear division. Initial cleavage is superficial. The cells do not become separated from the yolky interior of the blastula until the second day of development. At this time the outer cell membranes of the blastula are still covered with 15 μm spines.

TABLE I

Development, settling and metamorphosis.

Day	Event	Cultures					
		1	2	3	4	5	6
1	Fertilization and early cleavage	start*					
5	Gastrulation and cilia formation		start*				
10	Planulae begin swimming			start*			
15	Septa forming, planulae able to change shape, adults begin releasing planulae						
20	*Phyllochaetopterus* added		yes	yes			
25			+	+			
30			+	+	start^x		
35			++	++		start^x (dense)	
40	8 tentacles on settled anemones able to ingest *Artemia nauplii*		yes	yes			start$^{\circ}$
45	*Phyllochaetopterus* added	yes				+	yes
50	12 tentacles on settled anemones	++	++	++		+	++
75		+++	+++	+++		++	+++
125		+++	+++	+++	++	+++	+++

* Removed from the gastrovascular cavity of a female.

^{x} Put in culture after being released by a female.

$^{\circ}$ Subcultured from number 5.

Settling and metamorphosis: + = a few; ++ = many; +++ = most.

Gastrulation begins on day 3. The endodermal cells arise by multipolar ingression. Some cells may move into the endoderm by epiboly as in *Tealia crassicornis* (Chia and Spaulding, 1972), although the yolk plug structure reported for *Tealia* is not as prominent. As in the case of *T. crassicornis* the endoderm is difficult to define in histological preparations because of the presence of the yolk mass. At this time cilia begin to appear between the bases of the spines. At first they are sparse, but become more numerous within a few days. As the cilia develop the spines begin to shorten. By day 7 the spines are completely gone and the cilia completely cover the embryo. The beating of the cilia is uncoordinated at this time.

Gastrulation is complete by day 10 and the ciliary beating becomes coordinated. These planula larvae begin to move in the gastrovascular cavity of the female. Their movements become progressively more vigorous. They become more generally dispersed throughout the gastrovascular cavity, but the greatest concentrations are still in the tentacles and pseudospherules.

By day 15 longitudinal depressions are visible on the surface of the planulae, indicating the formation of the septa. The planulae begin to change shape. At this time they average about 1000 μm long by 600 μm in diameter. They may, however, contract into spheres or assume numerous other shapes.

On day 15 the female holding the nonfertilized eggs released most of them. On day 18 the two females brooding planulae began releasing them. The planulae were released from the mouth in small clumps of mucus which soon dispersed and the planulae swam away (Fig. 3d). By day 30 these females were releasing large numbers of planulae. Most of the planulae were released by 75 days.

Larval Settlement

Initially all culture jars contained a few stones; the sides of the jars gradually became covered with algae during the course of the observations. On day 20, tubes containing the polychaete *Phyllochaetopterus* sp. were added to culture jars 2 and 3 (Table I). The planulae began to settle and undergo metamorphosis in these two jars by day 25. In the other culture jars the planulae did not begin to settle until day 35 and then in fewer numbers than in jars 2 and 3. On day 45 *Phyllochaetopterus* tubes were added to culture jar 1 in which no planulae had settled. Soon after this the planulae began to settle in this jar in large numbers. The best settlement of planulae in jars without the *Phyllochaetopterus* tubes occurred in jar 5 in which the density of the planulae was approximately double that of the other jars.

Between days 35 and 40, the planulae that settled formed the eight primary tentacles and were able to capture and ingest *Artemia* nauplii. By day 50 these young anemones had developed 12 tentacles.

DISCUSSION

The eggs of *C. fernaldi* are similar to those described for *Tealia crassicornis* (Chia and Spaulding, 1972) and other anemones (Spaulding, 1974; Siebert, 1973; Dewel & Clark, 1974). The sperm were not observed in any detail.

We do not have any information concerning the time and site of fertilization. The eggs may be fertilized prior to their release from the gonads as is the case in *Actinia equina* (Chia and Rostron, 1970) or they may be fertilized in the gastro-vascular cavity after release from the gonads.

The embryonic development follows a course very similar to that of *Tealia crassicornis* and which has been described in greater detail (Chia and Spaulding, 1972).

Chia and Spaulding (1972) reported experiments with the effect of various substrata on the settlement of *T. crassicornis* planulae. Of the various materials tried, the tubes of *Phyllochaetopterus* were most effective. As in the case of *T. crassicornis*, a specialized substrate was not necessary for the planulae to settle, but appeared to hasten the process. High densities of planulae also appear to promote settlement in *C. fernaldi*.

Of special interest in the development of *C. fernaldi* is the brooding behavior. The eggs of this anemone are large and yolky. The developing larvae in culture were not fed until after settlement and metamorphosis. Sections of early planulae show a solid core of yolk filling the gastro-vascular cavity. Sections of planulae 20 days later, when they are capable of settlement, show much less yolky material remaining. Embryos and larvae removed from the adults at various times and put in culture developed at the same rate as those which were allowed to remain in the females. The larvae are lecithotrophic and do not appear to derive any nutritional benefits from brooding.

The brooding behavior may be protective in function, greatly reducing the loss of larvae by predation. The active part of the brooding is played by the females which retain the eggs and larvae. This is a more simple situation than that of *Actinia equina* in which the early embryos, perhaps at the morula or blastula stage, leave the female, spend a period of time in the plankton and then re-enter adults to complete their development. Larvae of *A. equina* do not seem to develop without this brooding (Chia and Rostron, 1970). Appellof (1900) reported that *T. crassicornis* brooded its larvae while Chia and Spaulding (1972) showed that what is presumed to be the same species, living in a different area, does not brood its young.

We classify the type of brooding behavior exhibited by *C. fernaldi* and possibly by *T. crassicornis* as facultative brooding. In these anemones it is conceivable that certain environmental conditions may promote brooding behavior, or it may be a matter of chance that the females retain the fertilized eggs. Another possibility is that we are in fact dealing with two species in the case of *T. crassicornis.* The answer to this question awaits further study.

We would speculate that the facultative brooding behavior exhibited by *C. fernaldi* and possibly *T. crassicornis* represents an intermediate step in the process of evolution that led to the more complex brooding behavior exhibited by *A. equina.*

Hand (1966) suggested that Actiniarians evolved from the Madreporaria by the loss of skeleton forming ability. He further suggests that the Actiniarian family Actiniidae is most closely related to the corals, citing similarities in the cnidoms and the sphincter muscles and the heavy symbiotic algal associations characteristic of these anemones. Development and larval behavior are also similar with internally brooding planulae being found in both groups (Hyman, 1940). We would like to see more work done on the early development and larval behavior of other members of the Actiniidae and the corals. Information thus obtained may help in the evaluation of the hypothesis that the Actiniidae is the anemone family most closely related to the corals. In addition, considerably more information is needed on the cnidom characteristics and taxonomy of the Anthozoa, particularly members of species in which different types of development have been reported.

SUMMARY

Cribrinopsis fernaldi is described as a new species of sea anemone from the San Juan Archipelago, Washington. This species is distinguished from the other members of the genus by the presence of approximately 96 tentacles and 96 pairs of mesenteries arranged hexamerously. The cnidom is distinctive and consists of spirocysts, basitrichs, microbasic *p*-mastigophores, and atrichs.

During March, freshly collected specimens of *C. fernaldi* spawned in the laboratory. The female anemones retained their eggs in the gastrovascular cavity and fertilization occurred internally. The embryos developed in the cavities of the tentacles and pseudospherules.

Some embryos were removed from the tentacles of the adult on day 1 (early cleavage), and day 7, day 13 (planula), and day 34. These continued their development and metamorphosed and settled at the same time as the larvae which remained in the adult until natural release. It is concluded that the brooding behavior is protective rather than nutritive in function.

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NITROGEN METABOLISM AND TROPHIC INPUT IN RELATION TO GROWTH IN FRESHWATER AND SALTWATER *SALMO GAIRDNERI*

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The downstream migration of juvenile members of the *Salmo* genus is occasioned by characteristic morphological, physiological and behavioral alterations, by which stenohaline freshwater parr are transformed into euryhaline migratory smolt (Baggerman, 1960; Hoar, 1963; Fessler and Wagner, 1969), capable of ascending the estuarine salinity gradients to the coastal environments (Koch, Evans and Bergström, 1959; Houston, 1961). This transformation is of a seasonal and transitory nature, maximum euryhalinity coinciding with the onset of the migratory period. Saltwater tolerance is only retained if the seaward migration is completed. Fish that remain in fresh water at the end of the migratory period revert to a near stenohaline condition known as post-smolt until the following spring when they again transform to euryhaline smolt (Koch and Evans, 1962; Conte and Wagner, 1965). In addition, a certain size has to be attained before salmonids can reach their full seasonal hypo-osmotic regularity potential (Conte and Wagner, 1965). This possibly explains the narrow size distribution of 14–18 cm fork-length found in migratory *S. gairdneri* smolts (Mayer and Larkin, 1954; Wagner, Wallace and Campell, 1963).

The size-dependent, as opposed to age-dependent development of euryhalinity has stimulated speculation as to whether growth regulatory factors play a role in the maturation process of the euryhaline ionic regulatory systems in salmonids (Houston and Threadgold, 1963; Parry, 1960). Evidence also suggests continued regulation in an hyperionic environment is concomitant with increased growth rates. Landlocked salmonids are normally of a smaller size at maturity than migratory members of the same species (Ricker, 1938; 1940). This is possibly a direct environmental effect, since offspring of nonmigratory lacustrine parents grow as large as the sea-run type if given the opportunity to migrate to the sea (Foerster, 1947). Furthermore, under both natural and laboratory conditions, saltwater salmonid populations exhibit increased growth rates compared to those in fresh water (Canagaratnam, 1959; Falk, 1969). Such increased growth rates may result from higher food availability, environmental stimulation of food consumption, greater assimlation efficiency of consumed food, or increased retention of assimilated food under saline conditions.

The present study is designed to gain information on the differential growth rates. Specific growth rates and protein metabolism are investigated in relation to trophic input in freshwater smolt and post-smolt and saltwater fish.

Nitrogen balance (Birkett, 1969; Gerkin, 1971) is used to represent protein metabolism and is investigated at the two ration extremes of fully fed and starving fish to ascertain which levels of the food conversion process are affected by increased environmental salinity.

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MATERIALS AND METHODS

The experimental fish were yearling, hatchery-reared rainbow trout (*S. gairdneri*) from the Trent Valley Trout Farm, Derby, England. All experimental fish were between 30–40 g and over 15 cm fork-length, the minimum size requirement for euryhaline development during parr-smolt transformation (Conte and Wagner, 1965). The fish were acclimatized to an optimum growth of 12° C temperature (Swift, 1961), and to a day-night cycle of alternating twelve-hour periods for at least four weeks prior to experimentation. The saltwater fish were adapted to 75% sea water during their smolt stage. High mortality occurred with fish kept in 100% sea water over a prolonged period of time, whereas fish survived indefinitely in 75% sea water (cf. Parry, 1960). No attempt was made to determine the sex of the fish as there is little gonadal development in yearlings. It was assumed that the sex of juvenile trout would not influence their growth rate, as Brown (1946) found no apparent difference in growth of two-year old male and female trout.

During the experimental period the fish were kept in polythene tanks at a density of eight fish per 100 litres. They were separated from each other by clear perforated perspex partitions to allow individual feeding and to prevent injury due to aggressive behavior. At all times fish were fed EWOS No. 4, yearling trout pellets (Locke and Linscott, 1969). Each compartment was individually aerated to ensure maximum oxygenation, since food consumption and growth are known to decline with an appreciable decrease in oxygen level (Hermann and Warren, 1962). Water was recycled through carbon and synthetic cotton filters and completely changed once a week.

Specific growth rates, nitrogen assimilation and excretion rates of salt- and freshwater fish were measured from a period of April to May when the freshwater trout were in smolt condition, and again from July to August when the freshwater trout were post-smolts.

Specific growth rates

Saltwater and freshwater fish were both divided into four groups, each group receiving a different daily ration. Group A was fed *ad libitum*; group B, approximately 0.009 g of food per day per g initial wet weight of fish; group C, 0.0025 g food per day per g wet weight; and group D was starved for the duration of the experiment.

Fish were weighed individually at the beginning and end of the six week feeding period to assess their growth during this time. Prior to the weighings the fish were starved for three days to ensure that their alimentary tracts were empty (Windell and Norris, 1969). At the time of the initial and final weighings several fish were killed by spinal severance, to determine both their nitrogen content and dry weights.

Nitrogen excretion and assimilation

The semi-micro Kjeldahl method (Hawk, Oser and Summerson, 1954) was used for all nitrogen analysis, employing Jacob's (1959) catalyst with concentrated sulphuric acid and hydrogen peroxide. The ammonia released upon steam distillation was collected in dilute hydrochloric acid and estimated photometrically, at 480 μ after Nesslerization.

To ensure that all fish had the same recent nutritional history, individual fish were fed a 1.5% body weight ration for a ten-day period, prior to measurement of their nitrogen excretion at various times over the succeeding ten-day stravation period. The initial measurement commenced one hour after final feeding. Nitrogen excretion was measured by the increase in nitrogen content of a known volume of water in which the fish were kept for twenty-four hours. These measurements produced a range of nitrogen excretion values for individual fish from fed to starved conditions.

Measurement of nitrogen excretion for the first two days of starvation, when the fish continue to defecate, is somewhat problematical. Faeces encourage bacterial action and the soluble fraction of the faeces increases the nitrogen content of the water. In fresh water, bacterial action in the presence of faeces induces a twenty-five per cent reduction in soluble nitrogen over a twenty-four hour period. No such action was detected either in salt water or in non-contaminated samples. However, no corrective action was taken in freshwater fish, as their experimental feeding regime was effective in restricting defecation to the last three hours of the nitrogen excretion measurement period. Consequntly a maximum four per cent reduction of soluble nitrogen was possible during this time. Approximately 40–60% of the total faecal nitrogen content of individual fish was found to be water soluble. It was therefore necessary to estimate the degree of contamination for each individual fish on the first day of starvation, in order to get a more realistic measurement of their nitrogen excretion. After the twenty-four hour measuring period of the first day, the water in which the fish had been kept was filtered through double layered Whatman G.F.A. 7 cm glass fiber discs to remove the particulate faeces suspended in the water. The nitrogen content of both filtrate (TN) and particulate material (PN) was then measured. The nitrogen content of the particulate faeces was used to estimate the proportion of total soluble nitrogen content of the filtrate that could be attributed to faecal contamination (SN), using the following equation: $SN = Sf/Pf \times PN$, where SN equals the soluble faecal nitrogen contamination, PN equals the measure of nitrogen content of the particulate faeces, and Sf/Pf equals the ratio of soluble to particulate nitrogen in the whole faeces of individual fish, collected at the time of the assimilation experiments described later. Thus the corrected nitrogen excretion (EN) for day one, allowing for faecal contamination is $EN = TN - SN$, where TN is the total soluble nitrogen content of the filtrate.

The gut assimilation rates of individual fish were measured following the nitrogen excretion measurements, as the direct faecal collection caused some scale loss, and resulted in a certain amount of ionic and osmotic stress which may have adversely affected the nitrogen excretion results. On the tenth day of starvation, feeding was recommenced for a further seven days with a 1.5% body weight ration containing 1% chromic oxide. The chromic oxide acts as an indigestible non-absorbable substance to give a baseline from which assimilation can be measured. At the end of the feeding period faecal samples were collected directly from the fish by applying slight pressure with thumb and forefinger between the ventral fin and anus. Care was taken to ensure faecal samples were not contaminated with urine. The samples were dried at 75° C for two weeks and sub-samples were then taken for determination of nitrogen and chromic oxide content. The diet was analyzed for the same parameters. Chromic oxide content was determined by wet ashing with concentrated nitric acid and 60% perchloric acid and analyzed photometrically at 350 μ (Furukawa and Tsukahara, 1966). The apparent digestibility of assimilation was calculated from the formula of Maynard and Loosli (1962): digestibility $= 100 - 100 \times (Cr_2O_3 \text{ in diet}/Cr_2O_3 \text{ in faeces}) \times (\text{Nitrogen in faeces}/\text{Nitrogen in food})$.

Data compilation and analysis

Growth rate. Under controlled constant laboratory conditions growth is a multiplicative process which in the early stages of life follows an exponential curve (Brett, Shelbourn and Hoop, 1969). Growth was therefore expressed as specific growth rate, the instantaneous rate of gain in weight per unit weight: specific growth rate $= (1nW_2 - 1nW_1/t_1 - t_2) \times 100\%$, where $1nW_1$ and $1nW_2$ are the natural logarithms of the weight of individual fish at time, t_1 and t_2 respectively, t being expressed in days.

The relationship between specific growth rates and food consumption is presented graphically; consumption being calculated as mean per cent ration, based on the initial (w_1) and final (w_2) weight: mean per cent ration $= [\text{daily ration (g)}/(w_1 + w_2/2)] \times 100\%$.

In addition, the effectiveness with which fish are able to convert food into flesh is numerically expressed in the two forms of gross and net growth efficiency. Gross efficiency (K) is the ratio of specific growth rate (ΔB) to consumed food (C), whereas the net efficiency (N) is the ratio of specific growth rate (ΔB) to the ration consumed in excess of maintenance ration ($C - M$).

Nitrogen metabolism. The nitrogen metabolism of a feeding fish can be expressed as: Consumed N − Faecal N = Assimilated N = Retained N + Excreted N. This amount of nitrogen retained for growth is calculated from the measure consumption, assimilation and excretion rates.

Knowing the nitrogen content of the fish flesh, it is possible to calculate the nitrogen retention directly from measurements of specific growth rate values at the same ration.

Two numerical expressions of protein utilization with respect to growth in trout are obtained using the equations: (1), the efficiency of protein utilization for growth = (Protein N retained/Protein N absorbed) × 100% and (2), the gross efficiency = (N retention/N consumption) × 100%.

RESULTS

Specific growth rates

Specific growth rates relative to food consumption are very similar in saltwater and freshwater fish when measured from April to May (Table I, Fig. 1). Maintenance rations are identical, and gross and net efficiencies at maximum ration show no significant difference (Table II). However, the fresh water fish have a tendency to consume a greater amount when fed *ad libitum*, leading to a slightly higher

TABLE I

Statistical parameters of the specific growth results.

Mean ration level	Mean specific growth rate ΔB	Sample size n	Standard deviation	Standard error of the mean (s.e.m.)
Salt water, spring (Fig. 1)				
A. 1.385%	0.814%	8	0.148	0.052
B. 0.752%	0.535%	6	0.122	0.050
C. 0.238%	0.009%	8	0.084	0.030
D. 0	−0.459%	7	0.075	0.028
Fresh water, spring (Fig. 1)				
A. 1.708%	0.941%	8	0.143	0.051
B. 0.829%	0.583%	8	0.069	0.024
C. 0.251%	0.011%	8	0.046	0.016
D. 0	−0.415%	8	0.032	0.009
Salt water, late summer (Fig. 2)				
A. 1.412%	0.789%*	10	0.156	0.049
B. 0.668%	0.356%	7	0.115	0.044
C. 0.229%	−0.106%	7	0.068	0.026
D. 0	−0.434%	9	0.136	0.048
Fresh water, late summer (Fig. 2)				
A. 1.665%	0.587%*	11	0.154	0.046
B. 0.665%	0.410%	5	0.100	0.045
C. 0.220%	−0.051%	6	0.053	0.021
D. 0	−0.381%	7	0.162	0.061

* Test for significant difference between mean specific growth rates of seawater and freshwater summer fish at ration A. $t = 2.84, f = 19, P = >0.01$.

specific growth rate at this ration. In contrast, in July and August the specific growth rate of the freshwater fish is significantly lower ($P > 0.01$) at *ad libitum* ration (Table I, Fig. 2). Consequently, during this period, both gross and net efficiencies are higher in the *ad libitum* saltwater fish (Table II). No such differences are apparent at the lower ration sizes. Body water content is not a contributive factor to the growth rate difference at *ad libitum*, as there is no difference in the dry weight of saltwater and freshwater fish (Table II).

Nitrogen metabolism

The nitrogen excretion rates of both the freshwater and saltwater trout decline at a similar rate over the initial six days of the starvation period after which the rate of excretion stabilizes at a more basal level (Figs. 3 and 4). (All excretion values for day 1 have been corrected for faecal contamination.) However, the actual nitrogen excretion rates of the post-smolt freshwater fish (July to August) differs

TABLE II

Growth parameters of freshwater and saltwater trout.

	Specific growth rate at *ad libitum* ration ΔB	*Ad libitum* ration C per cent mean ration	Nitrogen input mg N/kg fish/day	Gross efficiency at *ad libitum* ration $K = \Delta B/C$	Maintenance ration M	Net efficiency at *ad libitum* ration $N = \Delta B/C-M$	Dry weight as % wet weight	Nitrogen content mg N/gm dry wt fish at *ad libitum* ration
Salt water, April to May	0.814%	1.38	533	0.59	0.25%	0.72	22.2%	111
Fresh water, April to May	0.941%	1.70	656	0.55	0.25%	0.65	22.9%	110
Salt water, July to August	0.789%	1.41	544	0.56	0.27%	0.69	21.7%	110
Fresh water, July to August	0.587%	1.66	642	0.35	0.25%	0.42	21.0%	108

significantly from those of the saltwater fish and freshwater smolts (April to May) in two respects. Initially, the total daily nitrogen excretion of the post-smolt is higher, yet by the time nitrogen excretion has reached basal level, the post-smolt excretion rate is lower than that of the other fish (Table III).

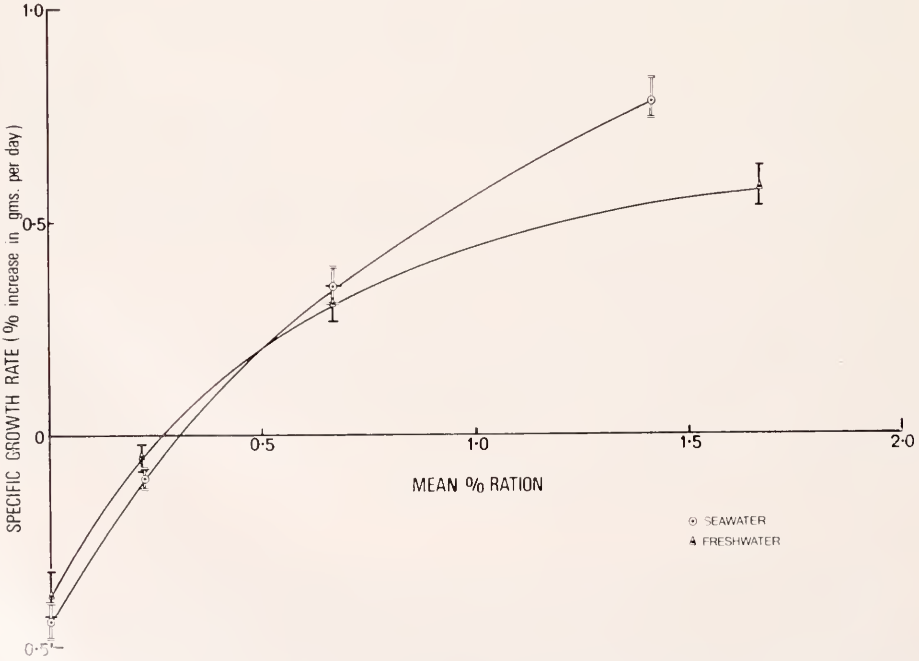

FIGURE 1. The relationship between specific growth rate and food consumption at 12° C for yearling rainbow trout during April to May.

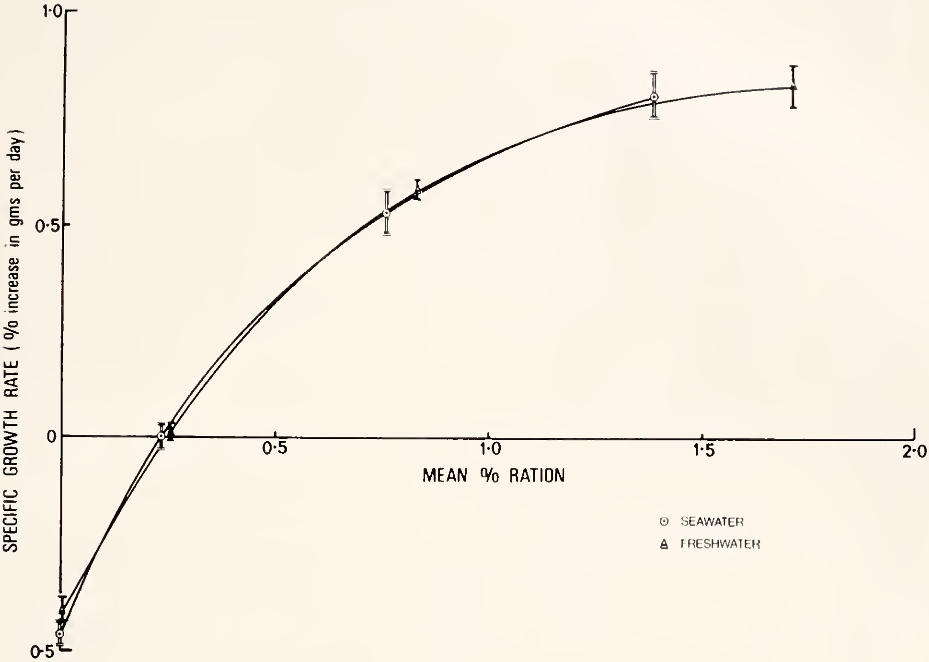

Figure 2. The relationship between specific growth rate and food consumption at 12° C for yearling rainbow trout during July to August.

Nitrogen assimilation

No differences are apparent in the gut assimilation of the freshwater smolt, post-smolt and saltwater fish. Mean assimilation rates were 77.1% in salt water and 78.2% in fresh water. Faeces inevitably include metabolic faecal nitrogen secreted

Table III

The nitrogen excretion on the first and tenth day of starvation of fed freshwater and saltwater trout.

	Number of fish	Day 1 nitrogen excretion (mg N/kg wet wt.) ($\bar{X}$ ± s.e.m.)	s.d.	*P* value compared with freshwater post-smolt	Day 10 basal nitrogen excretion (mg N/kg wet wt.) ($\bar{X}$ ± s.e.m.)	s.d.	*P* value compared with fresh-water post-smolt
Fresh water post-smolt, July to August	21	363 ± 13.6	62.5	—	60 ± 3	14	—
Fresh water smolt, April to May	13	316 ± 13.5	48.8	0.025	92 ± 5	17	0.001
Salt water, April to May	14	286 ± 10.8	41.8	0.001	97 ± 6	25	0.001
Salt water, July to August	12	287 ± 6.8	23.7	0.001	93 ± 9	30	0.001

s.d. = standard deviation; $\bar{X}$ ± s.e.m. = mean ± standard error of the mean.

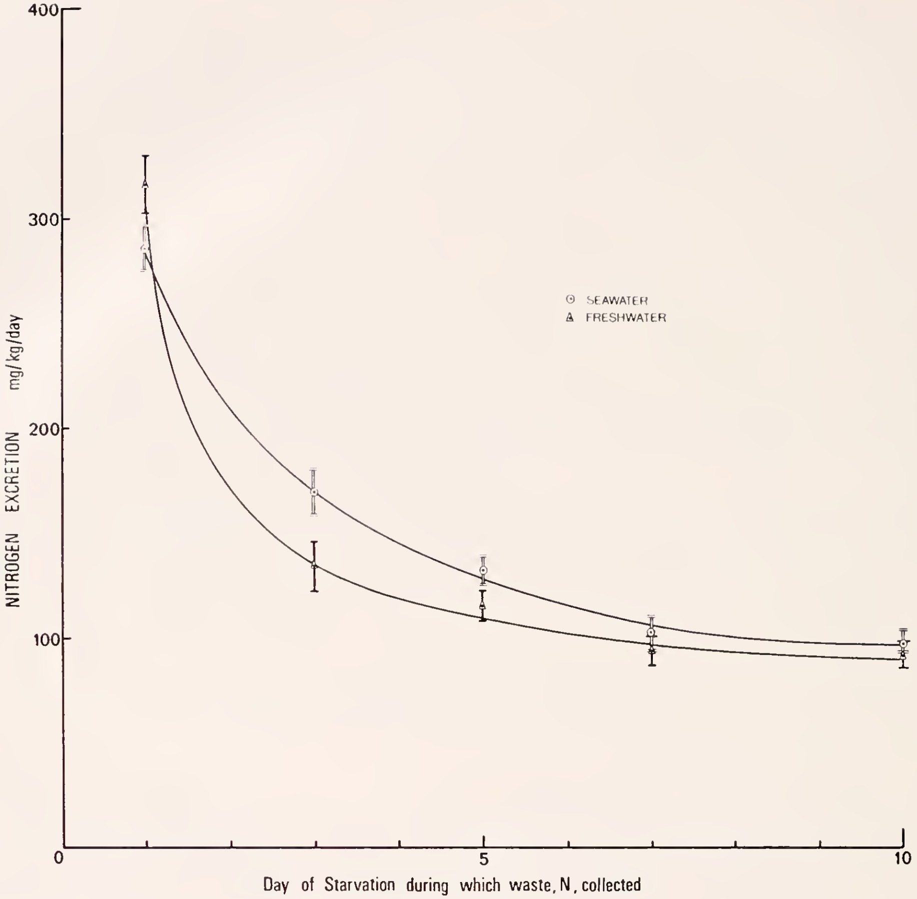

FIGURE 3. The nitrogen excretion of freshwater smolt and saltwater fish (April to May) over an initial ten day starvation period.

into the gut, which should not be considered as non-assimilated dietary nitrogen. Nose (1967) estimated that metabolic faecal nitrogen in rainbow trout amounts to 50–150 mg N/100 g of diet, which represents 5–17% of the total faecal nitrogen in

TABLE IV

The nitrogen retention efficiencies of freshwater and saltwater trout.

	Gross efficiency from nitrogen excretion	Efficiency of protein utilization	Gross efficiency from specific growth rates
Fresh water post-smolt, July to August	15.4%	19.7%	21%
Fresh water smolt April to May	23.4%	30.1%	36%
Salt water, April to May	27.3%	35.6%	33%
Salt water July to August	27.0%	35.4%	35%

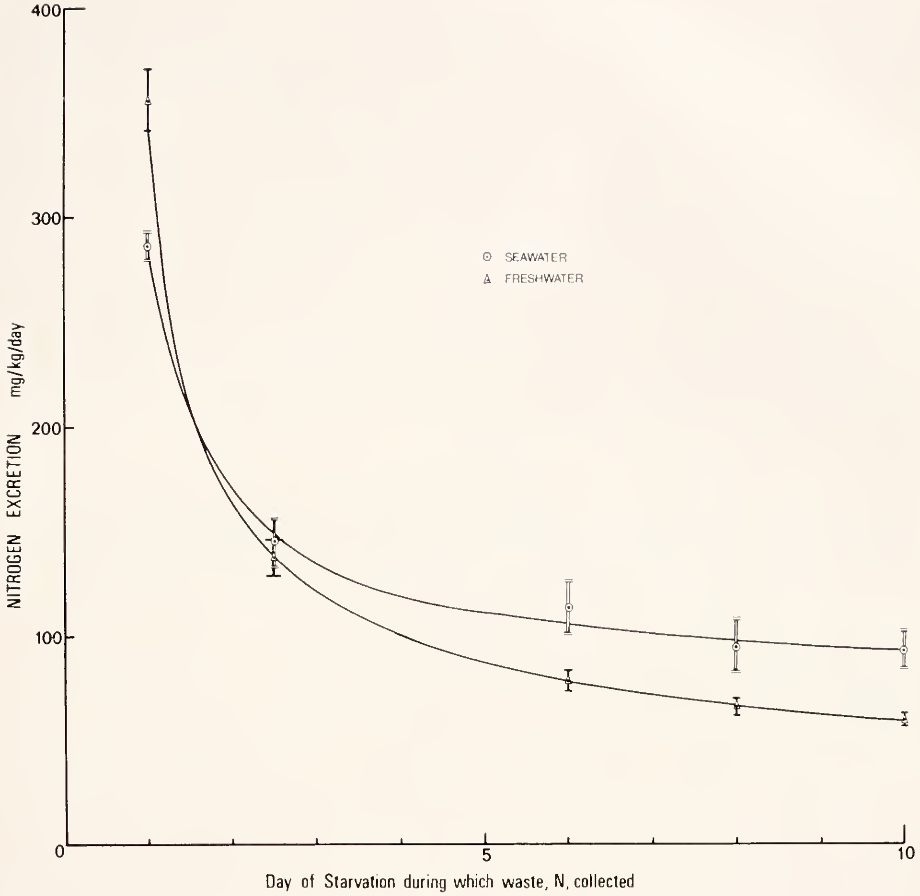

FIGURE 4. The nitrogen excretion of freshwater post-smolt and saltwater fish (July to August) over an initial ten day starvation period.

the present experiments. Taking the larger factor into account, the assimilation of the freshwater fish could be as high as 81.9% for the freshwater fish and 81% for the saltwater fish. This correction increases the proportion of excreted nitrogen by an amount equivalent to the increased assimilation. Values for nitrogen retention in the nitrogen balance equation are unaffected.

Nitrogen balance

A 1.5% ration gives a trophic input of 578 mg N/kg wet weight of fish/day, of which 452 mg N are assimilated in the freshwater fish and 446 mg N in the saltwater fish. If the nitrogen excretion values on the first day of starvation (Table III) are taken as representative of the nitrogen loss of a fully fed fish, then the difference between assimilation and excretion and the nitrogen retained for protein elaboration or growth is: 89 mg N/kg in the post-smolt freshwater fish (July to August), 136 mg N/kg in the smolt freshwater fish (April to May), 159 mg N/kg in the saltwater fish (July to August), 159 mg N/kg in the saltwater fish (April to May). Comparison between retention efficiencies based on excretion and specific growth rates (Table IV) show a discrepancy in both freshwater and saltwater fish. This could have arisen from the differences in experimental approach, or, more likely, from the difference in size distribution of the fish used in the excretion and growth experiments (40–60 g and 30–40 g, respectively), as Gerkin (1971) and Pandian (1967) have both reported decreased efficiency in protein utilization with increase in size in *Lepomis macrochirus* and *Megalops cypinoides*. This discrepancy does not alter the basic conclusion that the lower specific growth rate of the fresh water post-smolt at near maximum ration, compared to that of the smolt and saltwater fish, results from a decreased efficiency in nitrogen retention and not from differences in food consumption or assimilation. However, the lower basal excretion rate of the post-smolt (Table III) indicates an increased efficiency in conservation of body protein under the catabolic conditions of starvation.

Discussion

Previous reports on the relationship of growth to nitrogen metabolism in freshwater trout have given similar assimilation values, from 99.5% for milk caesin to 83.9% for egg albumin (Nose, 1967) and 80–90% for *ad libitum* egg white and liver (Atherton and Aitken, 1970). Although the physiological state of these fish was not given, their specific growth rate is comparable to the 0.94% rate of the present freshwater smolt as opposed to the lower 0.59% growth rate of freshwater post-smolt, both of which assimilated 78–81% of their 642–656 mg N/kg fish/day *ad libitum* ration. The seasonal change in *ad libitum* growth rate in freshwater trout under natural and constant temperature conditions has been previously described by Swift (1956) and Brown (1946). Brown (1946) attributed this change to an annual endogenous growth rate cycle, composed of an autumnal check, followed by rapid spring growth, slow summer growth, and finally another autumnal check. Swift (1961) correlated the cycle with seasonal environmental temperature change, which suggests a possible synchronization of the endogenous cycle with environmental temperature change. The present results show a close association between high specific growth rates and saltwater tolerance in trout. In fresh water the rapid spring growth of the cycle is coincident with the euryhalinity of the smolt phase, whereas the slower summer growth rate occurs at the time when the freshwater trout are in a stenohaline post-smolt condition. No such change in growth rate is apparent in the saltwater fish which continue to grow at an elevated rate throughout the year.

Under the same immediate experimental conditions and feeding regime, higher growth rates result directly from increased ability to retain assimilated nitrogen, and not from increased food availability, consumption or assimilation. The reciprocal nature of nitrogen excretion to specific growth rate in fully fed fish is seen in the seasonal fluctuation of excretion levels in fresh water and the continuous low rate of excretion in salt water, indicating an increased protein anabolic capacity in the fully fed freshwater smolt and saltwater fish. The basal nitrogen excretion of starved fish also show a seasonal fluctuation associated with the euryhalinity-stenohalinity cycle in fresh water which is not apparent in salt water.

However, in contrast to fully fed fish, the basal nitrogen excretion of starved fish suggests an increased protein catabolism in the freshwater smolt and salt water fish; post-smolt fish having a lower demand on their protein reserves under starvation conditions. The increased rates of synthesis in fed fish and degradation in starved fish may represent an overall increase in protein turnover that under maximum trophic input leads to higher specific growth rates.

Brown (1946) postulated the increased spring growth rate in the freshwater trout was endogenously induced by variation in the activity of the endocrine system. The close association between the increased protein metabolism and saltwater tolerance suggests that physiological induction of euryhalinity in freshwater fish simultaneously stimulates protein turnover and therefore growth. If the smolts remain in fresh waters past the migratory period, their return to stenohalinity is concomitant with decreased protein turnover. However, if the smolts do migrate to the sea, their ionic regulatory system is activated, tolerance to salt water maintained, and protein turnover stimulated further.

Smolt transformation and salt water tolerance are accompanied and most likely induced by thyroid, intrarenal and somatotrophic activity (Baggerman, 1960; Maetz, 1968; Olivereau, 1954). The hormonal induction and control of saltwater tolerance may possibly be the endogenous activity required to produce the annual growth rate cycle in freshwater and the sustained growth in saltwater *S. gairdneri.*

SUMMARY

If growth is considered over the complete annual cycle, saltwater trout grow at a greater rate than freshwater trout when fed *ad libitum.* However, during the spring, the growth rate of the freshwater smolt increases to a level equivalent to that of the saltwater fish. This is not primarily a result of differences in temperature of the natural seawater and freshwater environments, nor is it associated with differences in food quality, consumption or assimilation rates, but results from the increased rates of protein synthesis and degradation found in freshwater smolt and saltwater fish. The close association of the increased rates of protein metabolism with the smoltification process in freshwater fish, the continuation of this increase in the saltwater fish and the decrease found in the post-smolts remaining in fresh water, all suggest that the processes which bring about the transformation of a stenohaline freshwater trout to a euryhaline trout increase its rates of protein metabolism. It is known that various hormones play a role in the salinity adaptation of the migratory salmonids. Thus the possibility exists that such hormonal activity, primarily linked with osmotic and ionic regulation, may have a secondary effect on the protein metabolism and specific growth rate of the migratory rainbow trout.

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EFFECTS OF ENVIRONMENTAL FACTORS ON REGENERATION AND MOLTING IN FIDDLER CRABS

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Brachyuran crabs can autotomize limbs at a preformed breakage plane in the basi-ischium, and subsequently regenerate them. The limbs grow in a folded position and at ecdysis they unfold and become functional. The progress of limb regeneration has been divided into stages by Bliss (1956). After an initial lag period, basal growth occurs which establishes the primary organization of the limb (Hodge, 1956). This may be followed by a plateau of anecdysis if environmental conditions are not suitable. The next stage, one of intensive growth and further development of existing tissues, is the proecdysial stage, culminating in ecdysis and unfolding of the new limb. A terminal plateau often occurs just prior to ecdysis. Regenerating limb buds of crabs are generally described in terms of the regeneration index, or "R-value" (Bliss, 1956), which is (limb bud length/carapace width) × 100. Use of the R-value facilitates comparisons between crabs of different sizes.

Growth of the regenerating limb is related to the molt cycle of the crab. Factors that influence the molt cycle can influence the rate of regeneration, and vice versa. The molt cycle of Brachyura has been divided into five major stages by Drach (1939). Regenerating limbs have been used as an index to the stage of proecdysis for crabs, since the growth of limb buds is closely correlated to the progress of proecdysis.

The neurosecretory and hormonal systems exert important controls over the molt cycle. For example, in some species of crabs, ecdysone will accelerate proecdysial regenerative growth, leading to molt (Passano and Jyssum, 1963). Basal growth was found to be independent of the hormone. Removal of eyestalks, which contain molt-inhibiting hormones produced by the X-organ, will lead to accelerated regeneration and early ecdysis. Removal of many limbs (multiple autotomy) will also accelerate regeneration and molting in many species of crabs (Skinner and Graham, 1972). Fingerman and Fingerman (1974) have shown that removal of as few as two limbs will accelerate ecdysis in fiddler crabs.

The neurosecretory system, which controls the molt cycle, is itself affected by the external environment. Bliss and Boyer (1964) found that in the land crab, *Gecarcinus lateralis,* darkness, moderate temperature, and solitude were necessary environmental factors to permit proecdysial growth. These factors are likewise those which would increase the crab's chances of surviving ecdysis. Light, high temperature, and the presence of another crab inhibited proecdysial growth. The presence of dry sand delayed but did not stop regeneration. Rao (1965) found that light did not inhibit proecdysial growth in the ghost crab, *Ocypode,* if crabs were on a light background, and that privacy was a critical factor only for larger crabs, smaller individuals completing regeneration and molting in the presence of another crab. He also found that low temperature (15° C) lengthened the lag period but permitted basal growth in small individuals. Larger crabs did not initiate even basal growth. Passano (1960) found that low temperature inhibited basal growth as well as proecdysial growth in the fiddler crab, *Uca*.

The experiments described in this paper were designed to study the effects of various environmental factors, including light, starvation, privacy, temperature, salinity, and substrate on regeneration in *Uca*. In most cases regeneration was studied after multiple autotomy.

MATERIALS AND METHODS

Fiddler crabs, *Uca pugilator*, were collected from Accabonac Harbor, near East Hampton, New York. In one experiment, specimens of *U. pugnax* collected from Raritan Bay, near Union Beach, New Jersey, were used. In the laboratory, crabs were maintained with a small amount of sea water in community tanks (16 × 27 cm) or in individual containers. Crabs were used in experiments within two weeks of collection.

Autotomy was induced by pinching the merus with scissors. Multiple autotomy consisted of removal of one chela and six walking legs, and was performed in all experiments but one in which autotomy consisted of removal of only the first walking leg. Groups of ten to twelve animals of approximately the same carapace width were subjected to different environmental conditions after autotomy. In group situations, individuals could be differentiated by marking the carapace with nail polish. Crabs were fed Purina "Fly Chow" twice weekly.

Limb buds were measured at regular intervals under a dissecting microscope with an ocular micrometer. In all cases the limb bud measured was that of the first walking leg, and the values obtained were converted into R_1 values. To determine the statistical significance of the data, mean R_1 values for control and experimental groups were compared by a *t*-test. Times of molting were recorded for all animals.

RESULTS

Privacy and light

The combined effects of privacy and light were studied in a series of experiments.

Males. In a preliminary experiment five males (carapace width averaging 16 mm) kept in individual containers were compared with five in a group, all after multiple autotomy. Temperature was 19–22° C, and photoperiod was 14 hours of light to 10 hours of darkness. The results of this experiment are shown on the top of Table I. Initially, the limb buds of the isolated animals were larger than those of the grouped ones, but the difference was not statistically significant. Toward the end of the experimental period, the size of grouped animals' limb buds approximated that of the isolated ones. By day 28, two of the isolated but none of the grouped crabs had molted; but by day 30, three in each group had molted. Molting occurred at R_1 values of about 20.

The second experiment involved ten grouped and ten isolated individuals (carapace width 14–15 mm) exposed to a photoperiod of 14 hours light to 10 hours darkness (referred to as "light" below), and also ten grouped and ten isolated individuals of the same size maintained in darkness, all after multiple autotomy. The

TABLE I

R_1 *values (mean ± standard error) of* Uca pugilator *under differing conditions of light and privacy.*

	Days					
	7	11	14	18	21	24
Males						
Experiment 1—carapace width 16						
5 grouped	0.4 ± 0.1	1.5 ± 0.2	4.9 ± 0.3	8.9 ± 0.3	14.1 ± 0.5	17.6 ± 0.3
Isolated	0.5 ± 0.2	2.9 ± 0.7	5.8 ± 1.1	9.7 ± 1.2	14.7 ± 1.3	17.4 ± 1.1
Experiment 2—carapace width 14–15						
10 grouped light*	0.2 ± 0.1	1.7 ± 0.3	4.3 ± 0.5	8.1 ± 0.6	10.7 ± 0.7	12.9 ± 0.9
Isolated light*	0.6 ± 0.2	2.7 ± 0.5	6.7 ± 0.6	11.5 ± 0.9	15.0 ± 0.9	17.4 ± 0.7
10 grouped darkness	0.6 ± 0.2	1.9 ± 0.2	4.3 ± 0.6	9.6 ± 0.9	13.3 ± 1.0	16.5 ± 0.9
Isolated darkness	0.9 ± 0.2	2.9 ± 0.5	7.7 ± 0.7	11.1 ± 0.9	14.4 ± 1.1	16.1 ± 1.2
Experiment 3—carapace width 12–13						
Darkness		3.1 ± 0.2	10.3 ± 0.6	15.2 ± 0.8	18.9 ± 0.5	19.5 ± 0.4
Constant light		2.8 ± 0.3	10.2 ± 0.5	13.8 ± 0.8	17.2 ± 0.7	18.1 ± 0.6
Females						
Experiment 1—carapace width 15						
10 grouped	0.7 ± 0.2	2.4 ± 0.3	5.4 ± 0.5	8.6 ± 0.6	13.1 ± 1.0	16.8 ± 0.6
Isolated	0.6 ± 0.1	2.4 ± 0.3	5.6 ± 0.4	9.3 ± 0.6	13.3 ± 1.0	16.6 ± 0.9
Experiment 2—(1 leg removed) carapace width 14–15						
10 grouped light*	0.3 ± 0.1	0.6 ± 0.2	2.1 ± 0.5	5.4 ± 0.6	6.9 ± 0.8	8.7 ± 1.1
Isolated light*	0.3 ± 0.1	1.4 ± 0.3	3.2 ± 0.7	5.5 ± 0.7	7.1 ± 0.8	7.2 ± 1.0
10 grouped darkness	0.2 ± 0.1	0.8 ± 0.2	2.4 ± 0.5	4.8 ± 0.6	6.5 ± 0.9	7.7 ± 1.3
Isolated darkness	0.3 ± 0.1	1.6 ± 0.1	3.1 ± 0.5	4.7 ± 0.5	6.3 ± 0.6	6.9 ± 0.9
Experiment 3—carapace width 14–15						
10 grouped light*	1.3 ± 0.3	3.8 ± 0.5	9.8 ± 1.0	13.5 ± 1.1	17.4 ± 1.0	18.8 ± 0.7
Isolated light*	1.4 ± 0.1	5.0 ± 0.3	10.1 ± 0.6	14.4 ± 0.8	17.6 ± 0.6	19.2 ± 0.5
10 grouped darkness	1.3 ± 0.2	4.9 ± 0.2	9.4 ± 0.5	13.1 ± 0.7	16.4 ± 0.7	18.2 ± 0.4
Isolated darkness	1.0 ± 0.3	4.3 ± 0.5	9.2 ± 0.6	12.2 ± 0.8	16.1 ± 0.7	18.1 ± 0.6

* 14L/10D photoperiod

latter animals were exposed to dim light for a few minutes per week, during measurement and changing water. Results are shown in Table I. Starting at 11 days and continuing throughout the experiment the limb buds of the ten crabs in a group in light were significantly smaller than those of the isolated individuals in light. Molting was also retarded in the grouped animals in light. By 34 days 100% of the isolated but only 40% of the grouped animals had molted, an indication that grouping retarded, but did not prevent, regeneration and molting. Animals grouped in darkness initially regenerated at a rate that was not significantly different from that of grouped animals in light, but late in regeneration (about day 21) R_1 values of animals in darkness became significantly higher ($P = 0.05$). This would imply that the retarding effects of light became more evident as the crab approached ecdysis. The animals grouped in darkness molted sooner, with 90% having molted by 34 days, when only 40% of those grouped in light had molted. Animals isolated in darkness showed only a very transient period of time in which they regenerated more rapidly than animals grouped in darkness. Thus light seems to function as an inhibitory factor only in those animals that are in a group, and grouping (groups of ten but not five animals) seems to retard regeneration only in animals that are in the light. This is different from the situation in *Gecarcinus* in which the presence of another individual stopped regeneration altogether (Bliss and Boyer, 1964).

Since in this experiment "light" referred to a 14L/10D photoperiod, a third experiment was designed to ascertain if constant light could have a greater inhibitory effect, as Bliss and Boyer (1964) observed in *Gecarcinus*. A group of ten males, carapace width 12–13 mm, was placed in an environmental chamber in constant light and with a constant temperature of 20° C, after multiple autotomy. Control specimens were kept in darkness in the chamber within a light-tight box. Results are shown in Table I. Differences in rate of regeneration became apparent only toward the end of the regeneration period (as in experiment 2). Animals in darkness molted sooner and those in light exhibited a longer terminal plateau. At 25 days, 30% of those in darkness but none of those in constant light had molted. By 31 days, 100% of those in darkness, but only 70% of those in constant light had molted. Therefore, constant light did not seem to retard regeneration and molting to any greater extent than the 14L/10D photoperiod.

Uca, therefore, unlike *Gecarcinus,* continues proecdysial growth and will molt (with slight delay) in group situations and in constant light, even though many recently molted crabs are killed and eaten by their tank-mates, especially in groups with larger sized crabs. Even large males (carapace width 19–20 mm) proceeded to ecdysis in community tanks. Crabs undergoing ecdysis in community tanks were observed to move to the opposite end of the tank from where the other crabs congregated, thus increasing their chances for survival.

Females. The preliminary experiment involved ten crabs (carapace width averaging 15 mm) in a group and ten isolated at room temperature and 14L/10D photoperiod. The results are shown in Table I. No difference was seen in regeneration rate or time to ecdysis. By 30 days 50% of the grouped and 40% of the isolated crabs had molted. Thus it seemed that privacy had no effect on regeneration and molting in female fiddler crabs.

The second experiment involved the combined effects of isolation and light. Ten grouped and ten isolated individuals (carapace width 14–15 mm) were placed in the light (14L/10D photoperiod) and ten grouped and ten isolated individuals were placed in darkness. This is the same as was done with males except that in this experiment only one leg was autotomized. The results are shown in Table I. No consistent effects of light or grouping could be ascertained. Although at 11 days the isolated crabs had regenerated more than the grouped ones in both light and darkness, this difference subsequently disappeared. The low R_1 values are a result of data both from crabs that entered proecdysis and from those individuals that reached a plateau after basal growth and whose R_1 values remained below 10. No significant difference in number of crabs that entered plateau while in light or in darkness could be observed. Crabs were maintained longer than the table shows, with no striking changes. Plateaus, observed in this experiment after single leg autotomy, were not observed in crabs after multiple autotomy, in which growth was continuous. Ovigerous females, which were not included in experimental groups, delayed growth until eggs were gone.

The third experiment was a repeat of the second, except that multiple autotomy was performed. Results are shown in Table I. Again, no consistent effects of grouping or light could be observed.

Thus, there appears to be a sex difference in effects of isolation and light on regeneration in *Uca*, isolation being a factor only for males in the light, and light being a factor only for males in a group. The retarding effects of both grouping and light were rather small.

Temperature

To test the effects of lowered temperature, ten isolated and ten grouped males (carapace width 16–17 mm) were placed in an environmental chamber at a constant temperature of 16° C and a photoperiod of 14 hours light to 10 hours darkness. There was no indication even of basal growth in any crab by three weeks. Therefore, at this time the animals were returned to room temperature and used in a salinity experiment to be described below.

To see whether lowered temperature could block further regeneration and molting in crabs already in proecdysis, several male crabs with R_1 values of 15–20 were placed in the 16° C chamber. Growth continued slowly and most of these individuals did molt within a month, showing that ecdysis can take place at temperatures too low to permit basal growth and the initiation of proecdysis.

To test the effects of elevated temperatures, ten isolated male crabs (carapace width 14–15 mm) were placed in an environmental chamber at 30° C and a photoperiod of 12 hours light to 12 hours darkness. Controls at room temperature (20–23° C) were exposed to the same photoperiod. The animals at 30° C showed a very greatly accelerated regeneration rate. By one week they had R_1 values averaging 11.2 ± 1.9, whereas controls, which were just starting basal growth, had R_1 values of 1.6 ± 0.2. By 11 days, 40% of the crabs at 30° C had molted, and by two weeks 90% had molted. At this time controls had reached R_1 values of 9.4 ± 1.7. Only after 21 days had 40% of the controls molted. The size of the regenerated limbs of the animals at 30° C was normal despite the accelerated rate of growth.

Salinity

In all previous experiments the salinity of the water was 28–30‰. To test the effects of lower salinity, a group of male crabs (carapace width 16 mm) was placed in water that had been diluted with an equivalent amount of distilled water, yielding a salinity of 14–15‰. Another group was put at one-fourth of the normal salinity (7‰). The regeneration of these crabs was compared to that of similar crabs at normal salinity. Temperature was room temperature (20–23° C) and photoperiod was 14L/10D. Results are seen in Table II. The crabs at lowered salinities were delayed in the initiation of regeneration and therefore their limb buds were smaller than those of controls, but R_1 values approached those of controls toward the end of the regeneration period. Ecdysis occurred at about the same time, with 50% of the controls, 50% of the crabs at 15‰, and 70% of the crabs at 7‰ molting by 31 days.

To test the effects of hypersalinity, the crabs removed from the 16° C chamber were used. Upon removal from the cold, one group of ten was placed in water of one and one-half times normal salinity (45‰), made by adding NaCl to the water. The other group of ten served as controls at normal salinity. They were all at room temperature and a 14L/10D photoperiod. Five days after return to room temperature regeneration had begun in both groups. The data (Table II) show that limb buds of crabs at high salinity were significantly smaller than controls at all times. Molting was also retarded. Within 25 days after removal from the cold, 90% of the controls had molted. At this time none of the crabs at high salinity had

Table II

R_1 values (mean ± standard error) of U. pugilator *at different salinities.*

	Days					
	8	11	14	18	21	25
Males						
Experiment 1—carapace width 16						
Sea water	0.7 ± 0.2	3.0 ± 0.3	8.0 ± 0.5	11.3 ± 0.7	15.4 ± 0.6	17.5 ± 0.6
14‰	0.2 ± 0.1	1.6 ± 0.2	6.2 ± 0.5	9.8 ± 0.7	14.6 ± 0.9	17.3 ± 0.9
7‰	0.1 ± 0.1	0.7 ± 0.3	3.9 ± 0.8	7.1 ± 1.1	13.2 ± 1.4	16.0 ± 1.6
Experiment 2—carapace width 16–17 (returned to room temperature)						
Sea water	5.1 ± 0.3	8.9 ± 1.0	14.9 ± 1.6	19.1 ± 1.0		
45‰	1.7 ± 0.6	3.3 ± 0.6	6.0 ± 1.0	9.5 ± 0.9	12.0 ± 0.9	14.6 ± 1.0
Females						
Experiment 3—carapace width 15						
Sea water	2.4 ± 0.6	9.0 ± 1.2	13.0 ± 1.3	16.9 ± 1.8	20.0 ± 0.3	
45‰	1.2 ± 0.3	6.3 ± 1.1	8.6 ± 1.0	10.9 ± 1.8	14.8 ± 1.4	15.4 ± 1.2
15‰	1.1 ± 0.6	4.3 ± 1.1	7.3 ± 1.7	10.5 ± 2.2	14.9 ± 1.6	15.8 ± 1.4

molted, but by 35 days 90% of them had molted. It is possible that this response was influenced by their past experience at 16° C.

A third experiment was performed with groups of ten female crabs (carapace width 15 mm) at normal, 15‰, and 45‰ salinity. The crabs at 15‰ regenerated more slowly at the beginning, but subsequently regenerated at a faster rate than those at 45‰ salinity (Table II). Both groups remained significantly behind controls, however. By 21 days, 50% of the controls, but only 10% of the crabs at 45‰ and 20% of the crabs at 15‰ had molted.

Food

A group of ten males (16 mm carapace width) received no food after multiple autotomy. These were compared to fed controls. All were at room temperature (20–23° C), 28–30‰ salinity, and 14L/10D photoperiod. The starved animals lagged slightly but not significantly behind controls, and they molted at about the same time. At 31 days, 50% of the controls and 40% of the unfed animals had molted. Thus, *Uca* is able to regenerate and molt regardless of food intake during the period after multiple autotomy.

Substrate

Since fiddler crabs normally live in burrows in the sand or mud, regenerating individuals in their normal substrate were compared to crabs in water alone (at room temperature, 14L/10D photoperiod, and 28–30‰ salinity). For one experiment male specimens of *U. pugnax* (carapace width 15–16 mm) were used, with mud taken from their normal salt marsh habitat, as well as ample sea water. Crabs in mud were in separate individual containers, as were controls. Crabs in the mud dug burrows for themselves, and were much slower to initiate regeneration than controls (Table III). They were also much less active than controls. Later on, however, their limb buds grew rapidly and approached the size of the control limb buds. Crabs in mud molted almost as soon as controls, with 50% of the controls and 42% of those in mud molting by 21 days. Final R_1 values of the crabs in mud

TABLE III

R_1 values (mean ± standard error) of U. pugnax *in mud and in water.*

	Days			
	7	11	14	18
Males				
Carapace width 15–16				
Water	2.6 ± 0.5	10.0 ± 0.9	15.0 ± 1.4	16.7 ± 1.5
Mud	1.1 ± 0.4	5.6 ± 1.2	10.2 ± 1.7	13.2 ± 2.2

were lower than controls, but not significantly so. Sufficient water in the mud was necessary for survival through ecdysis.

The same experiment was performed with male specimens of *U. pugilator* (carapace width 15 mm) in their normal sandy substrate. There was at no time any significant difference in size of limb buds of these crabs and controls in water, and both groups reached ecdysis at the same time.

Re-regeneration

To test the readiness of recently molted crabs to resume the molt cycle and regenerate again, some newly molted female crabs (carapace width 15 mm) in stage B2–C1 were subjected once more to multiple autotomy. In a number of these, the autotomy reflex did not occur in some limbs. These crabs were not used in the experiment. Another group was subjected simultaneously to eyestalk removal as well as multiple autotomy, to see if these two methods of accelerating proecdysis could be additive. Mortality in the latter group was very high and all had died by two and one-half weeks, although during that time their regeneration rate was faster than that of controls with multiple autotomy alone. The experiment was repeated with somewhat better survival of the destalked crabs. Crabs with multiple autotomy and eyestalk removal regenerated at a faster rate than those with only multiple autotomy (Table IV). The regeneration rate of both these groups, however, was slower than that of crabs which had not molted just prior to experimental treatment. Furthermore, about one-third of the crabs with multiple autotomy alone reached a plateau after basal growth; these were the only crabs seen in all the experiments to have a plateau of anecdysis after multiple autotomy.

TABLE IV

Re-regeneration: R_1 values (mean ± standard error) of newly molted U. pugilator *after multiple autotomy (M.A.) or multiple autotomy and destalking (D).*

	Days					
	7	11	14	18	21	24
Females						
Carapace width 15						
M.A.	0.9 ± 0.2	4.5 ± 1.0	6.8 ± 1.3	8.9 ± 1.7	11.9 ± 1.3	13.8 ± 1.7
M.A. + D	1.6 ± 0.3	5.9 ± 0.9	10.3 ± 0.9	13.2 ± 1.7	16.1 ± 1.7	

In these re-regenerating crabs, both the right and left first walking legs were autotomized, whereas initially only the right first walking leg had been autotomized. This provided an opportunity to observe whether there might be a difference in regeneration of a limb which had just previously regenerated, and one which was removed for the first time. No difference was seen, however.

Observations on regenerated limbs

In all crabs except very small individuals (carapace width under 13 mm) the regenerated limbs were somewhat smaller than the original, both in length and thickness. Furthermore, regenerated limbs were much paler in color. A microscopic examination of *U. pugilator* revealed that the newly regenerated limbs contained about the same number of black and red chromatophores as did old limbs, but that these chromatophores were different in appearance. The chromatophores in the regenerated limbs appeared elongate and sparsely branched, so that they did not give a stellate appearance, even when expanded. It is conceivable that this may be due to a decreased dispersion of the pigment, due to differential response of these new chromatophores to the hormonal milieu. It is possible that they had not yet acquired sensitivity to the chromatophorotropins. It is more likely, however, that the cells had not yet developed the elaborate branching characteristic of mature chromatophores. Another morphological basis for the color difference between old and newly regenerated limbs was revealed. By removing eyestalks, all melanophores became punctate, and it could be observed that melanophores in the old limbs had more melanin and were approximately twice as large (0.05 mm in diameter) as those in the new limbs (0.025 mm diameter). Therefore, the pale color of the new limbs was due to the presence of less pigment as well as to a less branched state of the chromatophores.

After ecdysis, crabs which had regenerated seven limbs showed no significant increase in carapace width.

DISCUSSION

These experiments show that proecdysial growth in *Uca* is not stopped by light or grouping. These factors can only retard regeneration and delay molting somewhat under certain circumstances. Light can retard regeneration in males in a group, and grouping can retard regeneration in males in the light. The results of Bliss and Boyer (1964) with *Gecarcinus* are consistent with the ecology of that solitary burrowing land crab. *Uca*, although a burrowing crab, does emerge from burrows to forage in groups (Teal, 1958). The burrows of *Uca* frequently interconnect below the surface and they are more social crabs. It would be worth investigating whether there is a sex difference in *Gecarcinus,* since only males were used in Bliss and Boyer's (1964) study. The sex difference observed in *Uca* may be a reflection of greater aggressiveness on the part of male crabs, although an individual molting in a female community tank could get killed and eaten just as readily as one in a tank of male crabs. The response of *Uca* to light and grouping is somewhat closer to that of *Ocypode*. In that crab, juveniles were not inhibited from molting in the company of other crabs, and constant light could prolong the proecdysial period under some conditions (Rao, 1965). *Uca* is ecologically and phylogenetically more closely related to *Ocypode* than *Gecarcinus*.

The results of exposure to lowered temperatures confirm the results of Passano (1960) on *U. pugnax* after eyestalk removal, in which no regeneration took place at 15° C. He suggested that the cold blocked some metabolic processes necessary for basal as well as proecdysial growth. In *Ocypode*, Rao (1965) found that basal limb growth but not proecdysial growth could take place in juveniles at low temperatures, but adults did not even show basal growth. *Uca* is normally subjected to temperatures of 16° C and below during a large part of the year, and it might be suspected that crabs collected during the winter would be able to regenerate at low temperatures. This is not the case, however, since Passano (1960) collected crabs when the temperature was 12–15° C. The greatly accelerated regeneration at 30° C probably reflects the fact that the metabolism is higher at elevated temperatures. The initial lag period was shortened, and the rate of regeneration *per se* was greatly accelerated in these animals, which showed no signs of stress. This is in contrast to the results of Leffler (1972) on *Callinectes*, in which temperatures of 27° C and 34° C accelerated molting, but also increased the mortality rate at ecdysis. *Uca*, being an intertidal crab, would normally be exposed to warmer air temperatures during much of the summer.

The retardation of regeneration in crabs in hypo- and hypersaline water may be related to increased effort devoted to osmoregulation. It has been repeatedly demonstrated that the metabolic rate of crabs is elevated when they are subjected to unusually high or low salinities (Dehnel, 1960; Rao and Rao, 1963; Spaargaren, 1974). In the elevated temperature experiment, elevated metabolic rates were associated with accelerated regeneration and molting. Here, however, exposure to unusually high or low salinity, which also elevates metabolic rate, was associated with slower rates of regeneration. The extra energy being produced at high and low salinities was probably used for osmoregulation rather than growth. It is thought (Flemister and Flemister, 1951) that the increased metabolic rate is not due directly to increased osmotic work at the gills but to increased oxygen utilization by hydrated tissues, particularly muscle and hepatopancreas. Although salinity did not affect initiation of molting in the crab *Hymenosoma* (Broekhuysen, 1955), it has been shown to affect rates of growth and development of larval decapods, in which reduced salinity retarded growth (Costlow, Bookhout and Monroe, 1960). Metamorphosis was also delayed by high salinity (Costlow, 1967).

The lack of effect of starvation on regeneration confirms the results of Rao (1965) on *Ocypode* after limb autotomy. His experiment, however, was terminated before any crabs entered proecdysis. Apparently these species have enough food reserves to grow for that period of time without additional food intake. Roberts (1957) found that starvation inhibited molting in the crab *Pachygrapsus*. Vernberg (1959) and Wallace (1973) found that starved crabs (*Uca* and *Carcinus*, respectively) have a lower metabolic rate than fed crabs. The lowered metabolic rate which must have occurred in the present study did not have any significant effect on the regeneration rate. It is possible that starvation over a longer period of time would have had an effect.

It was initially surprising that *U. pugnax* initiated regeneration faster in water than in its own mud habitat. However, it should be mentioned that the area from which these crabs were collected is polluted, and the mud probably contained much that was deleterious to the crabs. It is also possible that toxic products accumulated in the containers since they were not flushed twice daily by tides as happens in the natural environment. If the slower regeneration were due to accumulation of toxic products, one would expect that the regeneration rate would be retarded later in the regeneration period as more material accumulated. However, the delay was due primarily to a longer lag period at the beginning of regeneration. Once regeneration had commenced in the mud, it proceeded fairly rapidly, and crabs reached ecdysis at about the same time as controls. The delay in initiating regeneration was not observed in specimens of *U. pugilator* in sand. It may be worth noting that the specimens of *U. pugnax* in individual dishes in clean water regenerated faster than any similarly treated *U. pugilator*. This may indicate another species difference.

It has been shown that re-autotomizing recently molted crabs results in a slower regeneration rate, due probably to the necessity to complete the shell hardening stages before the crab can begin to prepare for the next molt. Removing eyestalks as well as limbs can accelerate regeneration somewhat, but it results in high mortality and does not produce a rate of regeneration comparable to that exhibited by crabs which had not just previously molted. The pale color of newly regenerated limbs, as compared to old limbs, has been shown to be due to a sparsely branched condition of the chromatophores and to a smaller amount of pigment contained within them. This would imply that the pigmentation of the regenerating limbs is derived from new pigment cells, rather than migration of mature pigment cells from adjacent areas. This is the mechanism believed by Goodrich and Greene (1959) to occur in repigmentation of regenerating fish fins, and by Hsiang and Brick (1969) to occur in regenerating newt limbs.

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SUMMARY

After multiple autotomy, fiddler crabs (*Uca pugilator*) were exposed to a variety of environmental variables, during which time limb bud growth was measured and time of ecdysis was noted. The presence of other crabs retarded limb regeneration in males exposed to light. Light retarded limb regeneration in males in a group. Light and grouping had no effect on regeneration in females. Regeneration was totally inhibited at 16° C, but was greatly accelerated when the crabs were kept at 30° C. Exposure to unusually low (15‰ or 7‰) or unusually high (45‰) salinities retarded regeneration, primarily at early stages in the regenerative process. Starvation, however, had no effect on limb regeneration. Specimens of *U. pugnax* commenced regeneration more slowly in their normal mud habitat than in water alone, but *U. pugilator* regenerated at the same rate in water alone as in their normal sandy substrate. Regeneration of crabs autotomized shortly after ecdysis was much slower than that of crabs autotomized at a later stage in the molt cycle. Removal of eyestalks as well as limbs of such animals accelerated their rate of regeneration.

These effects on regeneration and molting are discussed with reference to the ecology of the fiddler crabs. The pale color of the newly regenerated limbs is due to a sparsely branched condition of the chromatophores as well as to the presence of a smaller amount of pigment within them.

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THE

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BURYING AND MOLTING OF PINK SHRIMP, *PENAEUS DUORARUM* (CRUSTACEA: PENAEIDAE), UNDER SELECTED PHOTOPERIODS OF WHITE LIGHT AND UV-LIGHT

Reference: *Biol. Bull.,* **150**: 163–182. (April, 1976)

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Photoperiod is known to affect the behavior and physiology of arthropods. Photoperiodism has been more thoroughly investigated in insects than in crustaceans, and it was not until 1946 that Panouse found that photoperiod may affect certain crustacean physiological processes. Later the effects of light on the molt cycle were examined by Bliss (1954a, b) and Stephens (1955), their work clearly demonstrating a relationship among photoperiod, hormonal regulation, and the molting process. Although a large number of papers discussing the effects of light on crustacean behavior and physiology have been published (Black, 1963; Buikema, 1968; Bulnheim, 1966; Lowe, 1961; Rao and Nagabhushanam, 1967), the effects of photoperiod on the molt cycle have received little attention, as noted by Aiken (1969).

The onset of molt in crustaceans is controlled by X-organ secretions of the molt-inhibiting hormone (MIH). Because the X-organ is located in the eyestalk (Passano, 1960), it may be light sensitive. Thus, if the MIH can be controlled by photoperiod, level of light energy, specific wavelength, or a combination of these, ecdysis may be induced. Results from Aiken's (1969) work on the freshwater crayfish *Orconectes virilis,* demonstrate that X-organ secretions are, in fact, controlled by various photoperiodisms.

During the initial studies of testing various pelletized foods for the shrimp, *Penaeus duorarum,* bacterial buildup became an increasingly important problem

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with time. An effort to reduce the bacterial population was made by irradiating different aquaria with selected photoperiods of UV-light. Results indicated that shrimp irradiated daily with zero, 12, and 24 hr of UV-light exhibited an increasingly higher molt rate, respectively. These preliminary data warranted further investigation, and a more thorough study was undertaken to describe the effects of different photoperiods of white light and UV-light on the burying, growth, and molting of *Penaeus duorarum* in controlled conditions. This paper presents and discusses the burying and molting data.

The diurnal burying behavior of *P. duorarum* has been reported by Eldred (1958), Eldred, Ingle, Woodburn, Hutton, and Jones (1961), Fuss (1964), and Ogren (1966), Idyll (1950), Viosca (1957), and Williams (1958). Wickham (1967) studied the persistence of the burying behavior under experimental conditions, and Hughes (1968) sought the factors that were responsible for the shrimp's emergence from the substrate. To date, however, their observations and conclusions have not been reinforced with long term experiments and sound statistical analysis.

MATERIALS AND METHODS

Shrimp used in the experiment were caught in shallow grass beds on August 21, 1969, midway between St. Teresa and Turkey Point, Franklin County, Florida, USA. The shrimp were placed in a seawater holding tank and selected for size and species. Pink shrimp, *Penaeus duorarum,* between 55 and 60 mm total length (*i.e.,* tip of rostrum to tip of telson) were used for the experiment because this size range allowed measurable growth rates over a period of several weeks and was also large enough to provide readily observed exuviae. Ten shrimp of similar size were placed in each tank. The shrimp were measured (total length) individually, and weighed in groups of ten, before being placed in aquaria and again at weekly intervals to determine growth rate.

Five groups of 38-liter aquaria (two/group) were exposed to different daily photoperiods: Group I received 0-hr light (00:24LD); Group II, 12-hr white light (12:12LD-W); Group III, 12-hr UV-light (12:12LD-UV); Group IV, 24-hr white light (24:00LD-W); Group V, 24-hr UV-light (24:00LD-UV). Aquaria of Group I (00:24LD) were placed in a black box (64×64×94 cm) to exclude all light (except during the monitoring periods), while the other groups were partitioned from each other by black cloth to prevent interference among the different photoperiods. Photoperiod refers to the light (L) and dark (D) cycle; photophase denotes the light phase, scotophase denotes the dark phase (Aiken, 1969). Photoperiods were controlled by an electric timer, and the photophase for Groups II (12:12LD-W) and III (12:12LD-UV) began at 1200 and ended at 2400 while the scotophase ran from 0000 to 1200. Ken Rad's fluorescent F 40 "Cool White Lamp" and General Electric's F-40-T-12-BLB "Black Light" lamp were used as a source of white light and UV-light, respectively. Spectral distributions of the lamps were obtained from the respective manufacturers. The spectral distribution of the white light was 400 to 700 nm; wavelengths of 520 to 660 nm contributed the most energy. The range of wavelengths for the UV-light was 300 to 400 nm. One lamp was placed approximately 30 cm above the water's surface and centered perpendicular to the long axes of the aquaria. The aquaria of each group were placed juxtaposed with their long axes parallel.

Feeding and observing the shrimp took place twice daily, once between 1300 and 1500 and again between 0100 and 0300. Initially, at each feeding, shrimp were fed chopped frozen squid amounting to 5% of the shrimp's total weight. After two weeks, however, shrimp showed poor growth so the amount of food was doubled and remained at this level for the duration of the experiment. Just prior to feeding, exuviae were collected, counted, and recorded. Presence of any dead shrimp was noted, and any excess food removed to prevent contamination.

Because the exact stage of the molt cycle was at first unknown, a minimum ot three exuviae per shrimp were examined to show the effects (if any) of the different photoperiods. Observations from preliminary studies revealed that approximately eight weeks are needed to obtain the desired number of exuviae for 55–60 mm shrimp (total length) ; hence, the experiments were continued for a duration of 56 days.

To ensure isotropic properties among the tanks, the water of the groups with an equal duration of photophase, *i.e.,* Groups II, III, and Groups IV, V, was interchanged. The water from the last tank in Group III or V was continuously pumped to the first tank in Group II or IV, respectively. Water circulated through the other tanks back to its original location *via* siphons, at flow rates approximating 75 liters/hr. Water was passed through under-gravel filters, and Silent Giant air pumps were used to supply aeration to the aquaria.

A two-cell flashlight (C-cell) with a red filter (> 550 nm) was used to observe the shrimp in Group I, and Groups II and III during the scotophase. Observations lasted less than 5 minutes a day for Group I and less than 2.5 minutes a day for Group II and Group III. Eldred (1958) found that red light caused least disturbance among active shrimp in the dark, and Fernandez (1965) found the maximum absorption of the eye pigments of *P. duorarum* to be at 516 nm. Growth rates were determined each week by measuring individual shrimp to the nearest mm (total length) and by weighing the shrimp in groups to the nearest hundredth of a gram. A triple-beam balance was used to obtain wet weights of the shrimp by placing them in a tared beaker of sea water and taking a weighted difference. In addition to the red flashlight, the shrimp in Group I were exposed to a maximum of 15 minutes of red light ($\lambda = 550\text{–}750$ nm) from an incandescent lamp once a week while they were being measured and weighed. The energy levels of the light used were measured by a YSI-Kettering Radiometer Model 65 with a YSI 6551 probe. The measured light energies at 30 cm were 2600, 100, 20000, and 16000 ergs $cm^{-2}sec^{-1}$ for the white light, UV-light, red filter and flashlight, and red incandescent light, respectively. Per cent transmission of light through 10 cm of aquaria water at the experiment's end was measured to be the following average values: white light (500–700 nm), 97; UV-light (300–400 nm), 85; red filter and flashlight, (> 550 nm), 98; and red incandescent light (550–750 nm), 98.

A Beckman-DK-2A ratio recording spectrophotometer was used to measure the transmission of light through the red filter and the aquarium water, and a Beckman DB-G grating spectrophotometer was used to obtain the transmission of light through a piece of the red incandescent lamp.

Temperature and salinity were maintained at 25° C and 25‰, respectively, because these conditions were found to be optimal for growth and survival of the brown shrimp, *P. aztecus* (Zein-Eldin and Aldrich, 1965), which is found in temperature and salinity ranges similar to those of *P. duorarum*. About 4 cm of sand-gravel mixture were used for a substrate because *P. duorarum* preferred this to other bottom types (Hildebrand, 1954; 1955; Williams, 1958).

Burying, growth, molt, and mortality data for the individual aquaria of each group were summarized into four two-week periods and an analysis of variance employing a split-plot arrangement in a completely randomized design was computed (Steel and Torrie, 1960). The two-week period burying, molt, and mortality data consisted of the cumulative number of shrimp above the substrate, number of exuviae, or mortalities divided by the cumulative number of shrimp in the tank at each observation, respectively. The effects of the treatments (*i.e.*, photoperiod), the periods (two-week interval), and the treatment × period interactions on average weight increases were evaluated. In a similar manner, the effects of the treatments, periods, times, and the treatment × period, the treatment × time, the period × time, and treatment × period × time interactions were also evaluated for the average burying, molt, and mortality rates. If a significant difference was found, then orthogonal comparisons (Snedecor and Cochran, 1967) were made to explain more specifically the difference among the treatments, periods, times, etc.

Throughout the text, references are made to shrimp being more active during the entire "photophase" or "scotophase," but observations lasted only a small fraction of these times, and in reality, cannot be extended to the entire time intervals. It is believed, however, that the data are indicative of the influence of the photoperiods on shrimp behavior in aquaria or culture conditions, but care should be exercised in interpreting the results and conclusions to wild populations of shrimp subjected to many additional and interacting variables. For convenience, the photoperiod is frequently given in parenthesis behind a particular group. The terms *treatment* and *group* are used synonymously.

Environmental factors

Initially it was not known whether the wavelength, the amount of energy, the photophase, or a combination of these factors affected the burying behavior, growth, and molt rate of shrimp. Because pink shrimp usually bury themselves during the photophase (Fuss, 1964), it is difficult to estimate how much energy, if any, they are receiving. The shrimp in groups with continuous light were not always buried and therefore were exposed to some light. Sometimes the shrimp buried themselves completely, while at other times their antennal scales, eyes, and rostrum were exposed. Therefore, the energy received by a shrimp is dependent upon its burying behavior.

Ideally the intensity of the lights should have been such that the groups with an equal photophase also received equal amounts of energy, so any difference found between these groups could be attributed only to the difference in wavelengths. Shrimp exposed to equal photophases, however, were not irradiated with the same amount of energy because the equipment to do so was not available. In any event, if a significant and similar response was observed in both groups irradiated with

UV-light and not in the white light controls, then the difference would be attributable to wave-length rather than photoperiod or energy.

Available light energy at the bottom of the aquaria was estimated by substituting the needed values into the equation expressing the extinction of radiation in sea water, *i.e.*, $I = I_0 e^{-kz}$ (Neumann and Pierson, 1966). The aquarium water depth (z) was 21 cm, the incident radiation (I_0) is given in the text, the absorption coefficient (k) was estimated by examining the spectral distributions of the lights used and converting the average percent transmission of the aquaria water for the wavelengths which contributed the most to absorbance ($A = \log T^{-1}$). The daily amount of energy received at the surface of the substrate in the aquaria was calculated to be approximately 11, 0.66, 22, and 0.74 joules cm^{-2} for Groups II, III, IV, and V, respectively. The energy received by Groups III (12:12LD-UV) and V (24:00LD-UV) is similar because the flashlight used during scotophase monitoring for Group III contributed almost as much energy as 12 hr of UV-light. Group I (00:24LD) received about 0.6 joules cm^{-2} daily and an additional 1.4 joules cm^{-2} once a week while measuring the shrimp's growth. The energy calculations for Group I are absolute maximums because the flashlight was on for no more than five minutes each day and not directed at any individual shrimp during that time.

The pH of the water was initially 8.2 and dropped to 7.2 for Groups I, IV, and V; and to 7.0 for Groups II and III. This is not considered to have a deleterious effect, as it is in the range of estuarine environments inhabited by the species at this age.

RESULTS

Burying

The weekly cumulative number of shrimp counted above the substrate in each aquarium for each observation time is presented in Table I, and Table II presents the corresponding weekly cumulative number of shrimp in the aquaria. An

TABLE I

Weekly cumulative number of shrimp above substrate.

Group	I				II				III				IV				V			
Tank	1		2		3		4		5		6		7		8		9		10	
Time**	p	s	p	s	p	s	p	s	p	s	p	s	p	s	p	s	p	s	p	s
1st week	46	46	40	46	02	61	04	63	01	57	01	49	19	28	18	34	08	08	07	06
2nd week	48	46	31	45	09	49	12	68	00	56	09	62	12	28	25	31	15	06	26	10
3rd week	50	50	48	59	03	55	01	58	01	57	03	66	05	15	09	23	14	26	16	21
4th week	47	53	54	45	05	59	02	45	00	61	00	62	14	19	26	26	28	21	17	22
5th week	43	52	46	54	06	45	02	36	03	60	00	54	21	26	20	17	05	12	05	14
6th week	44	45	42	42	03	43	02	32	01	45	01	42	15	09	15	20	05	12	09	12
7th week	33	44	39	37	07	39	01	30	03	29	04	32	11	13	21	23	12	07	12	11
8th week	29	29	25	27	04	40	03	29	01	18	04	33	07	06	12	26	09	15	10	19

** p = 1200–2400, s = 0000–1200.

TABLE II

*Weekly cumulative number of shrimp.**

Group	I				II				III				IV				V			
Tank	1		2		3		4		5		6		7		8		9		10	
Time**	p	s	p	s	p	s	p	s	p	s	p	s	p	s	p	s	p	s	p	s
1st week	70	70	70	70	70	70	70	70	70	70	70	70	70	70	70	70	70	70	70	70
2nd week	70	70	70	70	66	67	70	70	66	67	70	70	70	70	70	70	70	70	70	70
3rd week	70	70	70	70	63	63	62	64	63	63	70	70	70	70	63	63	70	70	65	65
4th week	70	70	70	70	63	63	56	56	63	63	66	67	70	70	63	63	65	66	63	63
5th week	68	69	69	69	59	60	53	54	63	63	58	59	64	64	61	61	63	63	60	60
6th week	61	62	63	63	56	56	44	44	59	60	56	56	60	61	55	56	54	57	51	52
7th week	54	55	56	58	56	56	40	41	39	39	50	51	37	38	46	47	42	42	41	42
8th week	43	43	33	37	46	47	32	33	24	24	42	43	28	28	38	38	31	32	33	33

* The average number of shrimp per day in any tank for any given week may be obtained by summing the cumulative number of shrimp observed during the two observation times for that tank and week and dividing by 14.

** p = 1200–2400, s = 0000–1200.

ANOVA of the number of active shrimp per total shrimp is given in Table III. Significant differences were found to occur among the treatments, periods, times, period × time, treatment × period, treatment × time, and the treatment × period × time interactions. The mean number of shrimp above the substrate for the sources of variation are presented in Table IV.

An average of 69% of the shrimp of Group I (00:24LD) were visible above the substrate. This was significantly greater than the 24 to 44% average observed to be active in Groups II–V. Shrimp in Groups II and III were significantly more active (43%) than those of Groups IV and V (28%). The burying behavior of shrimp irradiated with a 12-hr photophase (Groups II and III) did not differ significantly, nor was there a significant difference between that of shrimp irradiated with 24-hr light (Groups IV and V).

The burying behavior of *P. duorarum* during the first and second two-week periods was not significantly different from that of the third and fourth two-week periods. Significantly more activity, however, was observed during period 2 (45%) than period 1 (41%), and highly significantly more activity was observed during period 4 (45%) than period 3 (40%).

Significantly more burying occurred from 1200–2400, *i.e.,* during the time coinciding with the photophase of Groups II and III. The average number of shrimp, for all groups, observed above the substrate from 1200–2400 and 0000–1200 was 25 and 59%, respectively.

Because mean shrimp activity was similar during the periods 1 and 3, and 2 and 4 (see period section of Table IV), the treatment × period interactions were analyzed individually for lunar influence rather than for the effects of the passage of time. That is, significance was tested for differences between periods within each treatment (see Table III). The orthogonal comparisons consisted of three tests for each treatment: periods 1, 3 *vs.* 2, 4; period 1 *vs.* 3; and period 2

TABLE III

ANOVA for cumulative number of shrimp above substrate (Table I) per cumulative number of shrimp (Table II).

Source of variation	df	Mean square
Treatment	04	0.45686**
T_1 *vs.* T_2–T_5	01	1.36949**
T_2, T_3 *vs.* T_4, T_5	01	0.39843**
T_2 *vs.* T_3	01	0.00090
T_4 *vs.* T_5	01	0.05863
Error a[1]	05	0.01073
Period	03	0.01366**
P_1, P_2 *vs.* P_3, P_4	01	0.00060
P_1 *vs.* P_2	01	0.01704*
P_3 *vs.* P_4	01	0.02333**
Time	01	2.32917**
Period × time	03	0.00629*
Treatment × period	12	0.01261**
T_1:P_1, P_3 *vs.* P_2, P_4	01	0.00914
T_1:P_1 *vs.* P_3	01	0.01307*
T_1:P_2 *vs.* P_4	01	0.00190
T_2:P_1, P_3 *vs.* P_2, P_4	01	0.00034
T_2:P_1 *vs.* P_3	01	0.01636**
T_2:P_2 *vs.* P_4	01	0.00193
T_3:P_1, P_3 *vs.* P_2, P_4	01	0.00024
T_3:P_1 *vs.* P_3	01	0.00014
T_3:P_2 *vs.* P_4	01	0.01223*
T_4:P_1, P_3 *vs.* P_2, P_4	01	0.00000
T_4:P_1 *vs.* P_3	01	0.00523
T_4:P_2 *vs.* P_4	01	0.02988**
T_5:P_1, P_3 *vs.* P_2, P_4	01	0.10155**
T_5:P_1 *vs.* P_3	01	0.00011
T_5:P_2 *vs.* P_4	01	0.00013
Treatment × time	04	0.60607**
T_1(00:24LD)	01	0.00868
T_2(12:12 LD-W)	01	2.21377**
T_3(12:12 LD-UV)	01	2.49134**
T_4(24:00 LD-W)	01	0.03543*
T_5(24:00 LD-UV)	01	0.00421
Treatment × period × time	12	0.00639**
Error b	35	0.002098
Total	79	

[1] Aquaria per treatment.
* Significant at $\alpha = 0.05$.
** Significant at $\alpha = 0.01$.

vs. 4. In Treatment I, significantly less activity occurred during period 1 (62%) than period 3 (70%); in Treatment II, highly significantly more activity occurred in period 1 (48%) than 3 (39%); in Treatment III, significantly more activity occurred in period 2 (48%) than 4(40%); in Treatment IV, highly significantly less activity occurred in period 2 (26%) than 4 (38%); and in Treatment V, highly significantly less activity occurred during periods 1, 3 (15%) than 2, 4 (31%). Difference for all other comparisons were not found to be significant.

TABLE IV

Mean shrimp above substrate.

Source of variation		Observations (n)	Mean shrimp above substrate
Treatment	I (00:24LD)	16	0.69
	II (12:12LD-W)	16	0.44
	III (12:12LD-UV)	16	0.43
	IV (24:00LD-W)	16	0.32
	V (24:00LD-UV)	16	0.24
Period	1 (26 Aug–09 Sept)	20	0.41
	2 (10 Sept–23 Sept)	20	0.45
	3 (24 Sept–07 Oct)	20	0.40
	4 (08 Oct–21 Oct)	20	0.45
Time	1200–2400	40	0.25
	0000–1200	40	0.59

Treatment × period*	I	II	III	IV	V
26 Aug–09 Sept	0.62	0.48	0.42	0.35	0.15
10 Sept–23 Sept	0.73	0.46	0.48	0.26	0.31
24 Sept–07 Oct	0.70	0.39	0.43	0.30	0.16
08 Oct–21 Oct	0.69	0.43	0.40	0.38	0.32
Treatment × time**					
1200–2400	0.66	0.07	0.04	0.28	0.22
0000–1200	0.71	0.81	0.83	0.37	0.25

* Number of observations (n) = 4.
** Number of observations (n) = 8.

The activity of the treatment × time interaction was found to be significantly different. Activity was highly significantly greater from 0000–1200 for Groups II and III, and significantly greater for Group IV. The mean number of shrimp visible per number of shrimp present during 1200–2400 and 0000–1200 was 0.07 and 0.81, 0.04 and 0.83, and 0.28 and 0.37 for Groups II, III, and IV, respectively. No significant differences were found between the times for Groups I and V (Table IV).

Orthogonal comparisons for the significant differences found for the period × time and the treatment × period × time interactions were not computed, because the time factor is primarily responsible for their differences.

Molting

The weekly cumulative number of exuviae collected in each aquaria for each observation time is presented in Table V. Table VI is an ANOVA of the cumulative number of exuviae per cumulative number of shrimp (Table II). Tables VII, VIII, and IX give the mean molt rate for the periods, the times, and the treatments × time interactions, respectively.

Highly significant differences were found among the periods. The molt rate was significantly greater during the second period (4.2%) than the first (2.5%) and during the latter two periods (4.0%) compared to the first two periods (3.3%).

TABLE V

*Weekly cumulative number of exuviae.**

Group	I				II				III				IV				V			
Tank	1		2		3		4		5		6		7		8		9		10	
Time**	p	s	p	s	p	s	p	s	p	s	p	s	p	s	p	s	p	s	p	s
1st week	0	2	0	1	4	0	6	0	2	0	1	0	0	0	0	0	6	0	3	0
2nd week	0	4	0	3	5	1	3	0	5	0	4	0	2	0	5	2	4	0	6	1
3rd week	4	1	6	1	5	0	6	0	8	0	5	0	4	3	1	6	3	3	5	3
4th week	2	1	1	0	6	0	4	0	5	0	4	0	4	4	3	2	4	1	2	2
5th week	3	1	7	1	6	0	4	0	5	0	6	0	1	3	1	3	1	5	3	3
6th week	5	1	2	1	4	0	4	0	4	0	3	0	3	2	1	5	0	4	1	2
7th week	2	0	5	1	7	0	3	0	3	0	4	0	1	1	1	2	2	2	2	2
8th week	4	0	4	1	2	0	3	0	2	0	3	0	2	1	0	3	1	0	0	1

* Exuviae found during the photophase probably resulted from ecdysis during the scotophase and vice versa.

** p = 1200–2400; s = 0000–1200.

No significant differences were found between the third and fourth period molt rates (Table VII).

Highly significant differences were found between times. From 1200–2400, the mean number of exuviae found per shrimp for 40 observations was 0.055; and that from 0000–1200 was 0.018.

TABLE VI

ANOVA for cumulative number of exuviae (Table V) per cumulative number of shrimp (Table II).

Source of variation	df	Mean square
Treatment	4	0.000139
Error a[1]	5	0.000085
Period	3	0.001191**
P_1, P_2 *vs.* P_3, P_4	1	0.000855*
P_1 *vs.* P_2	1	0.002714**
P_3 *vs.* P_4	1	0.000003
Time	1	0.026514**
Period × time	3	0.000317
Treatment × period	12	0.000189
Treatment × time	4	0.005824**
Group I	1	0.003648**
Group II	1	0.025297**
Group III	1	0.019937**
Group IV	1	0.000389
Group V	1	0.000537
Treatment × period × time	12	0.001123**
Error b	35	0.000143
Total	79	

[1] Aquaria per treatment.

* Significant at $\alpha = 0.05$.

** Significant at $\alpha = 0.01$.

TABLE VII

Mean death and molt rate per period.

Period	Observations	Mean death rate	Mean molt rate
1	20	0.001	0.025
2	20	0.002	0.042
3	20	0.008	0.040
4	20	0.018	0.040

The treatment × time interactions were found to be highly significantly different. The mean percent of exuviae found per shrimp for the times 1200–2400 and 0000–1200 was 4.8 and 1.8, 8.0 and 0.1, and 7.1 and 0.0 for Groups I, II, and III, respectively. No significant differences in the molt rate were found between the times 1200–2400 and 0000–1200 for Groups IV and V (Table VIII).

Orthogonal comparisons were not computed for the highly significant differences found among the treatment × period × time interactions because this difference was caused mainly by the difference in the treatment × time interaction.

DISCUSSION

The shrimp's activity above the substrate was inversely related to the amount of exposure to light. This is substantiated by the fact that shrimp were significantly more active in the absence of light (Group I) than those in the remaining groups, which were subjected to some light exposure, and significantly more active in groups irradiated with 12-hours of light (Groups II and III) than those exposed to constant light (Groups IV and V). The activity of the groups of shrimp exposed to equal durations of light, but different wavelengths, was similar (refer to treatment section of Tables III and IV). On the whole, most activity occurred from 0000–1200, *i.e.*, during the scotophase of Groups II (12:12LD-W) and III (12:12LD-UV).

TABLE VIII

Mean molt rate for treatment × time interaction.

Treatment	Time	Observations	Mean molt rate
Group I	1200–2400	8	0.048
Group I	0000–1200	8	0.018
Group II	photophase	8	0.080
Group II	scotophase	8	0.001
Group III	photophase	8	0.071
Group III	scotophase	8	0.000
Group IV	1200–2400	8	0.031
Group IV	0000–1200	8	0.041
Group V	1200–2400	8	0.044
Group V	0000–1200	8	0.033

TABLE IX

Number of exuviae per shrimp.

Group	I	II	III	IV	V
Total exuviae	64	73	64	66	72
Average number of shrimp per group for 56 days	18.06	16.25	16.64	16.73	16.46
Exuviae per shrimp for 56 days	3.54	4.49	3.85	3.94	4.37

More exact information about the time and treatment effects is obtained from the orthogonal comparisons of their interactions. The average burying behavior between 0000–1200 and 1200–2400 was not found to be significantly different for shrimp in Group I (00:24LD). That is, an endogenous rhythm of burying activity was not found to exist in shrimp kept in constant darkness. If additional data had been collected with 1.5 hours after sunrise or sunset, an endogenous rhythm may have been found, because evidence was obtained from Group I to support the existence of an endogenous molt rhythm with most molts being found from 1200–2400 (Tables V and VIII). Thus, the preclusion of light does not necessarily interrupt all rhythms. Wickham (1967) found a nocturnal circadian activity pattern for *P. duorarum* after 100 hours of continuous darkness, and Racek (1959) found that *P. plebejus* maintained a nocturnal activity pattern in constant darkness for a month. Dall (1958) found that *Metapenaeus mastersii* exhibited a nocturnal activity pattern in constant darkness for at least seven days. Neither Dall (1958) nor Racek (1959) gave information about the procedures for keeping the tanks dark or how the shrimp were observed. Fuss and Ogren (1966) kept *P. duorarum* in continuous darkness for three days and found the shrimp more active the entire time, but this activity declined with the passage of time. After several weeks to natural light acclimation, *P. semisulcatus* maintained distinct circadian burying activity for four days in constant darkness (Moller and Jones, 1975). They found the emergence of *P. semisulcatus* to be more closely synchronized with "expected" dusk than re-entry to "expected" dawn. No endogenous burying rhythm, however, was found for *P. monodon* acclimated and tested in a similar manner (Moller and Jones, 1975). Brown (1961) discusses other activity cycles of crustaceans held under the continuous absence of light.

The influence of light on the burying behavior is most evident in the treatment × time interaction of Groups II (12:12LD-W) and III (12:12LD-UV). Almost all activity exhibited by the shrimp occurred during the scotophase (0000–1200) with very little being observed during the photophase. Thus it appears that light is the most important factor governing activity of *P. duorarum* as reported by Fuss and Ogren (1966). Certainly the significant difference between the times 0000–1200 and 1200–2400 (see Tables III and IV) is due primarily to the activity differences found in Groups II and III. Hughes (1968) found that over 90% of the shrimp present emerged from the substrate within 45 minutes after the light-dark transition. He also found the light-dark transition to be the Zeitgeber for emergence, or at least a principal factor of the Zeitgeber. In addition, he found evidence of a 24-hour feeding rhythm. When food was made available immediately after the light-dark transition, the burying rhythm could be resynchronized to an eight-hour advance of the light-dark transition within four days. When food was not made available after the transition, resynchronization took six days (Hughes, 1968). Moller and Jones (1975) also found light to be the main synchronizer for activity for 24-hour periods. They monitored the activity patterns of *P. monodon* and *P. semisulcatus* irradiated with 06:18LD, 12:12LD, and 18:06LD photoperiods of white light, and found that the periods of activity were closely related to the scotophase. Similar results were obtained for *P. duorarum* which were subjected to a natural photoperiod and a photoperiod with a 6 hr advance of the scotophase (Wickham and Minkler, 1975).

An examination of the daily activity data shows that in the present experiment, shrimp of Groups II and III resynchronized almost immediately; during the first week, an average of over 88% of Group-II shrimp and 75% of Group-III shrimp were observed above the substrate during each monitoring time of the scotophase, and less than 2 and 4%, respectively, were observed during the photophase. Therefore, the change of the timing of the light-dark transition from that of natural conditions is considered to affect only the timing of the burying or emergence.

A circadian burying rhythm was found for shrimp continuously irradiated with white light (Table III, treatment × time section). Shrimp of Group IV (24:00LD-W) were significantly more active from 0000–1200, thus coinciding with the activity patterns of Groups II (12:12LD-W) and III (12:12LD-UV). Although the light conditions were constant throughout the experiment, there was a semidiurnal feeding pattern to which the shrimp could have adjusted for their burying pattern. But if feeding became the predominant Zeitgeber in the presence of constant light, a similar burying pattern would be expected to occur also in Group V (24:00LD-UV). Because a circadian burying pattern was not found in Group V, it might be possible that particular wavelengths of light are needed for the continuance of the circadian burying behavior or that continuous UV-light might interfere with a burying rhythm. Fuss and Ogren (1966), Wickham (1967), and Wickham and Minkler (1975) found evidence of circadian burying that coincided to natural burying for *P. duorarum* irradiated with continuous light, and Hughes (1968) found a circadian periodicity of burying for *P. duorarum* under a constant low light intensity after three consecutive days. Thus white light might be necessary for maintaining a circadian burying pattern.

The burying activity differences between the two monitoring times for Group IV (24:00LD-W) are obvious only during the first three weeks (Table I). Circadian rhythms are not always 24-hour cycles (Brown, 1973), and it is possible that the burying activity was changing from 0000–1200 to 1200–2400 during the later four to five weeks of the experiment (Table I). Wickham (1967) found that under constant dim light for 72 hours, activity peaks for *P. duorarum* occurred approximately one hour later each night and noted that these peaks "progressed daily with the tides." In the absence of natural tidal and solar rhythms, the circadian burying activity could have progressively diminished so that by the fourth week, the activity patterns were no longer evident. More frequent observations per 24 hours would be necessary to verify this.

No significant differences in activity were found between the first two periods compared to the last two periods. There was, however, significantly more activity in the second period compared to the first, and significantly more in period 4 compared to period 3. Thus, there appears to be little change in burying behavior for the overall experiment, but there are changes among the periods. An average of just over 40% of the shrimp was visible during both periods 1 and 3, and periods 2 and 4 both had an average of 45% visible. Periods 1, 2, 3, and 4 covered the intervals from August 26 to September 9; September 10–23; September 24 to October 7; and October 8–21, 1969, respectively. The full and last-quarter moon occurred during periods 1 and 3, and the new and first-quarter moon occurred during periods 2 and 4 (*American ephemeris and nautical almanac for the year 1969*). Overall, shrimp were most active during periods 2 and 4 (*i.e.*, during the new and first-quarter moon), but this difference was found to be present only in Group V (24:00LD-UV). Significant differences were mixed for the other interactions and are not easily explainable. Activity of Groups I (00:24LD) and II (12:12LD-W) differed between periods 1 and 3; shrimp were more active in Group I during period 3 and more active in Group II during period 1. Groups III (12:12LD-UV) and IV (24:00LD-W) exhibited activity differences between periods 2 and 4. Again opposite reactions occurred: most activity occurred in period 2 for Group III and in period 4 for Group IV. There seems to be no correlation for moonphase, photoperiod, energy levels, or wavelengths except for Group V.

In field studies, Fuss (1964) and Fuss and Ogren (1966) found that activity was less during periods of increased moonlight, though aquarium studies under continuous dark or light during the full and last-quarter moon phase revealed no strong evidence of lunar rhythmicity. Wickham (1967) found no apparent differences of burying behavior under constant conditions during the new and full moon, and Racek (1959) testing for lunar effects on the activity of three species of prawns in aquaria exposed to a natural photoperiod and continuous darkness for a month failed to find any differences. Previous studies of penaeid shrimp behavior and moon phase correlations (Beardsley and Iversen, 1966; Fuss, 1964; Fuss and Ogren, 1966; Racek, 1959; Wheeler, 1937; and Wickham, 1967) seem to support the conclusion that lunar phase exhibits an influence *in situ*, but that this influence is lost in aquaria studies. An exception is found in Aaron and Wisby's (1964) work in which a correlation was observed between moon phase and photoactivation of *P. duorarum* with maximum and minimum photoactivation being observed during the full and new moon, respectively. Apparently this is the only other reported evidence of possible lunar influence on behavior of *P. duorarum* in aquaria. The relationship between moon phase and shrimp activity for Group V indicates that there may be a lunar effect, but more controlled experiments are needed before any conclusions can be made.

The molt data obtained from this experiment did not reproduce that obtained in the preliminary study. The preliminary experiment, however, was only a cursory investigation, and it is believed that the molt data reported here are a truer indication of the effects of the selected photoperiods and wavelengths on the molting of shrimp.

Because Bliss and Boyer (1964) showed that light affects molting through the eye in at least one decapod crustacean (*Gecarcinus*) and because the X-organ, sinus-gland complex is associated with the eye, it is assumed that the

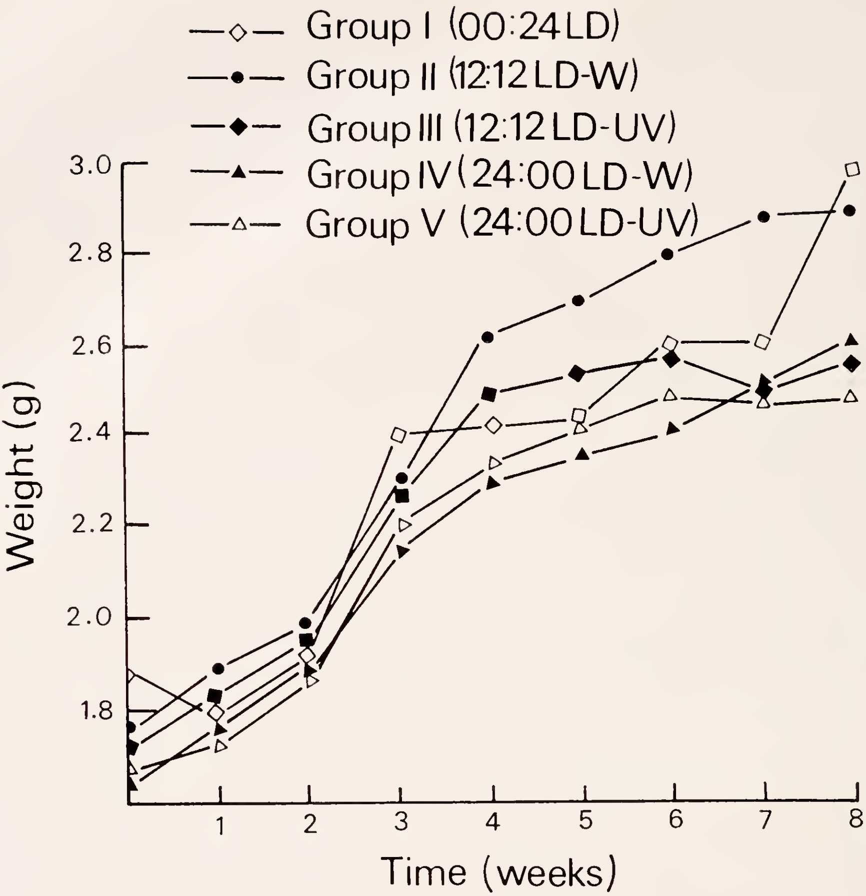

FIGURE 1. Growth (average weight).

MIH may be photoregulated (Aiken, 1969). Photoregulation of the X-organ may be achieved in one of four ways: by a particular wavelength, the amount of energy, the photoperiod, or through combined interactions.

The equivalent number of total exuviae among the groups indicated that the photoperiod, wavelength, or a combination did not markedly affect the rate of molting, but rather, influenced the time molting took place. Most exuviae were found from 1200–2400, but because monitoring of all the aquaria occurred not more than two hours after the scotophase-photophase change of Groups II and III, it is assumed that most molts took place from 0000–1200.

The mean molt rate is similar throughout the experiment (Table VII) except for period 1 during which the molt rate was lower than that in period 2. Presumably the lower molt rate was due to acclimation to laboratory conditions. Periods 1, 3, and 4 showed low growth rates (*i.e.*, 0.16 g *vs.* 0.52 g for period 2), but only period 1 showed low molt rates (Table VII). Therefore, either shrimp

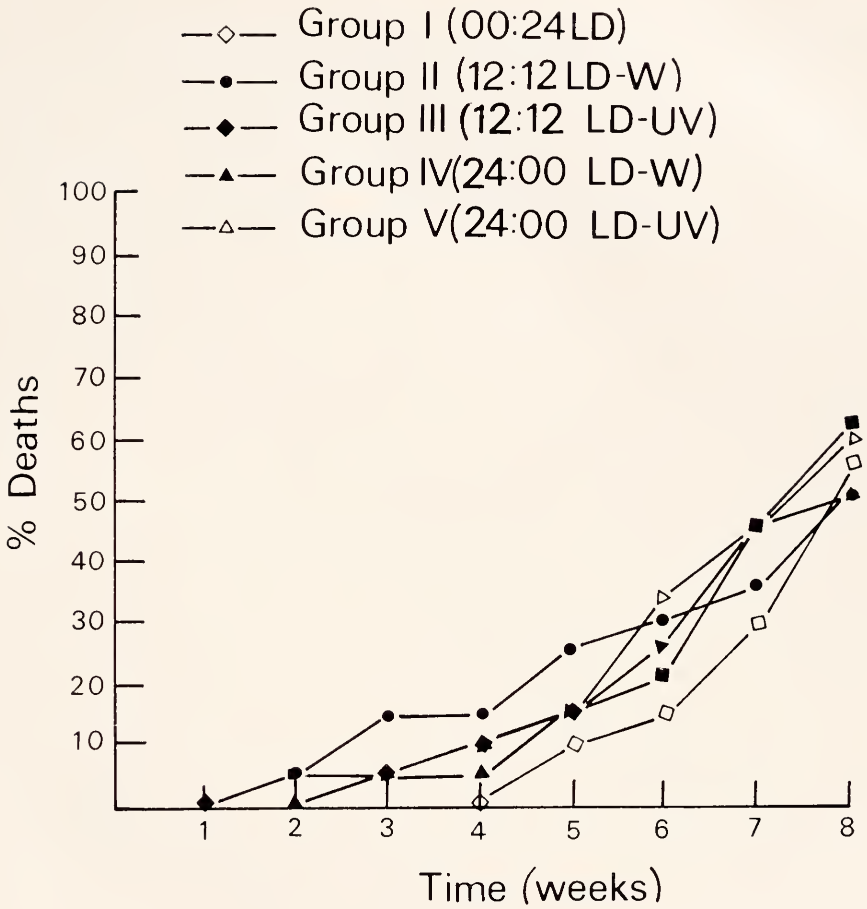

Figure 2. Cumulative per cent deaths.

were molting without growth, as reported by Eldred, *et al.* (1961) or the faster growing individuals were dying. The latter possibility is supported because mortality increased markedly during the period that the growth rate decreased (Figs. 1 and 2, Table VII). The possibility that the faster growing shrimp died during ecdysis is also supported by the results of Aiken's (1969) experiments, in which the freshwater crayfish, *Orconectes virilis*, died during ecdysis induced by alteration of the photoperiod. Similar effects may have occurred in the present experiment. On the other hand, the molt rate did not decline during this period, suggesting that the molt processes continued throughout the experiment (Fig. 3).

The high number of exuviae being found between 1200–2400 in Group I (00:24LD), indicates that *P. duorarum* may have an endogenous molt rhythm in the absence of light. Similar molt differences for the monitoring periods were not found in Groups IV (24:00LD-W) or V (24:00LD-UV), so continuous light

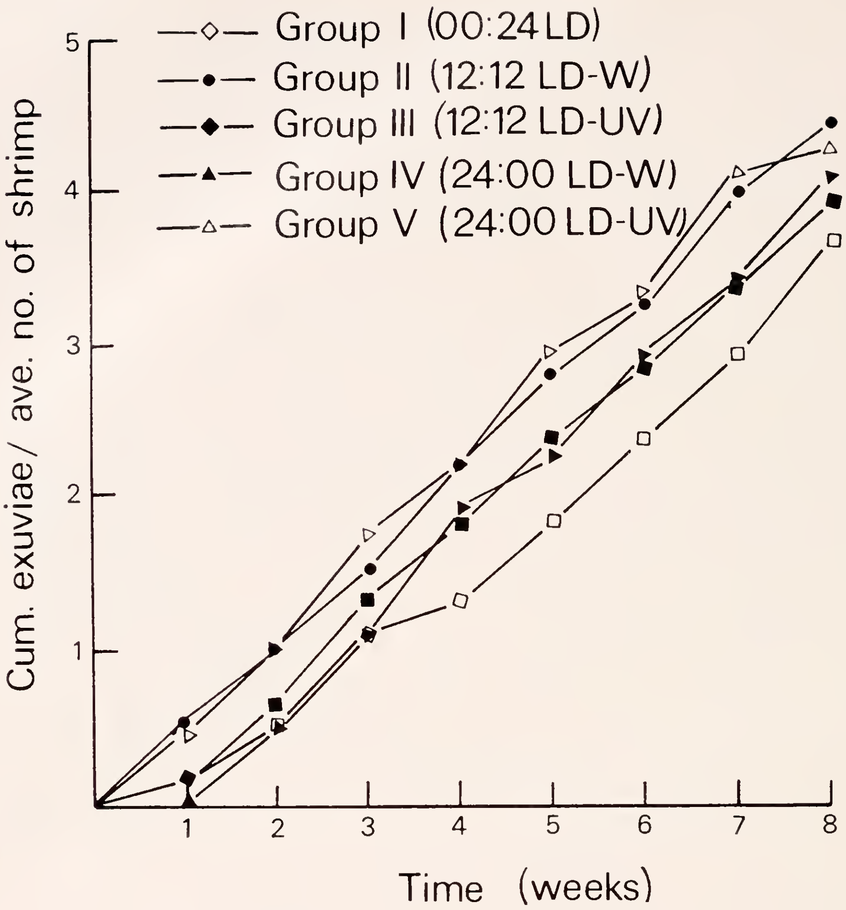

FIGURE 3. Cumulative exuviae per average number shrimp.

may interfere with this rhythm. A cursory examination of the daily exuviae data show no trends that would be negated by the two-week summation of the data. It cannot be determined from the present data whether the molt rhythm obtained for Group I is a fluke, but it should be pointed out that nearly twice as many exuviae were found from 1200–2400 than 0000–1200 in both tanks of Group I (see Table V). Dall (1965) reports that the molting of *Metapenaeus* sp. is inhibited in constant dark or constant light.

Groups II and III showed a highly significant difference of ecdysal activity between the two monitoring times. All exuviae except one were found during the photophase, 1200–2400 (Table V). Eldred (1958) and Dall (1965) found all molts to take place late at night or early in the morning. This nocturnal molting behavior probably serves a protective function as well as coinciding with the shrimp's activity. Shedding an exoskeleton is an extremely vivacious process

(Eldred, 1958) and would be difficult if not impossible to do while buried during the day. Newly molted shrimp are in their most vulnerable state and therefore darkness might offer some protection from predators that hunt by daylight.

Because there was no significant difference among treatments, an average molt rate was obtained by dividing the total number of exuviae by the average total number of shrimp (Table IX). This resulted in an average molt rate of one exuvium per 14 days for 55–73 mm shrimp (total length), approximately the rate mentioned in past studies. Known intermolt periods for penaeid shrimp range from 12–14 days (Dall, 1965; Eldred, *et al.*, 1961; Hudinaga, 1942), and for caridean shrimp from 13–36 days (Jefferies, 1964; Lloyd and Yonge, 1947; and Reeve, 1969). Nouvel-van Rysselberge (1937) found that the growth rate is less in aquaria conditions than in nature, thus a fortnightly exuviation in aquaria is probably less than that in natural conditions.

Because *P. duorarum* probably exuviates seasonally in its northern range, it may have MIH (see Carlisle, 1954). Therefore, one would expect the different photoperiods used to affect the molt rates. The average molt rates for the 56 days, however, were not significantly different (Tables VI and IX) and cumulative exuviae per average number of shrimp in different groups differ only slightly (Fig 3). Thus, the different lighting conditions affected the time of molting and apparently had little or no affect on increasing or decreasing the molt rate.

Generally, groups of shrimp exposed to an equal duration of light responded similarly, indicating that the shrimp were responding to a wavelength of light common to both bulbs used or that the length of the photophase, regardless of the wavelength, caused the response. Both the white light and the UV-light contain 320–400 nm wavelengths, and it may be this portion of the spectrum to which the shrimp were responding. Except for the endogenous activity differences found in Groups IV and V, the burying behavior as well as the molt rate were similar between groups with an equal photoperiod timing, *i.e.*, Groups II and III and Groups IV and V. It is believed, therefore, that the shrimp were responding to the length of the photophase (or scotophase) and not to the wavelength of light.

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SUMMARY

1. Shrimp activity was inversely related to the amount of light exposure.

2. Shrimp responded to a 12-hour photophase of white light and UV-light in a similar manner. The light-dark transition is apparently the Zeitgeber, and the burying rhythm can be readily changed with a change in the light-dark transition.

3. A circadian burying rhythm was found for shrimp kept in continuous white light; no rhythm was found for shrimp kept in continuous darkness or UV-light.

4. Evidence was obtained to support a lunar influence, with shrimp of Group V being more active during the new and first-quarter moon.

5. Burying activity between the first and last four weeks of the experiment was similar.

6. No significant differences among the groups' overall growth rates or molt rates were found for the four two-week periods.

7. The photoperiod influenced the time rather than the rate of molting. Groups II and III molted highly significantly more during the scotophase. Group I with no light molted highly significantly more during the time interval corresponding with the scotophase of Groups II and III, indicating presence of an endogenous molt rhythm.

8. Molt rate did not decline in the latter half of the experiment as did the growth rate.

9. Deaths approached 50% for all groups at the end of eight weeks.

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LARVAL SETTLING BEHAVIOR IN *CYANEA CAPILLATA* (CNIDARIA: SCYPHOZOA)

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The interactions between organisms and their environment are complex and reflect the adaptive response to a particular set of conditions by each stage in the life history. This is especially conspicuous in species with dissimilar life history stages, each with different demands on the environment. The success of such species requires that the adaptations of the immature forms lead to maturation by the adult in an environment suitable for successful reproduction. This can be a stringent requirement in species with a motile larva whose pattern of settlement effects the distribution of a sessile or sedentary adult; larval behavior adapted for the selection of substrate thus can maximize survival of the attached reproductive stage. This life history sequence, and the potential capacity for substrate selection, is characteristic of many marine invertebrates.

Numerous workers have analyzed marine larval behavior in the higher phyla, and they show that larvae recognize and select specific substrates with which their sedentary adult stages are associated. The most intensive studies deal with the larvae of polychaetous annelids, the pelecypod molluscs, and Cirripedia (Arthropoda). Recent reviews (Newell, 1970; Meadows and Campbell, 1972; Crisp, 1974) summarize this work, and also report studies on the Ectoprocta and Urochordata. These larvae respond to light, gravity, or current in a manner which leads them to the general vicinity where attachment can occur. Once in contact with the substrate, the larva reacts to surface texture and its chemistry. These characteristics are sorted by the larva during a behavioral sequence which leads to the selection on a firm substrate of a specific site for attachment; this sequence is similar among the diverse species studied (*e.g.*, Reese, 1964). Crisp (1974, p. 196) divides this searching behavior into three phases, the last of which is a period of "inspection", a minute examination of the substrate surface; if the stimuli received by the larva during inspection are appropriate, attachment follows. The knowledge of larval behavior in the higher phyla is sufficient to allow the formation of theoretical models of habitat selection (*e.g.*, Doyle, 1975).

The extent to which the larvae of lower phyla select specific sites for attachment requires investigation to determine whether these fit the pattern of behavior shown by more complexly organized larvae typical of the higher phyla. The planula larva of the Cnidaria, a motile (stereo-) gastrula (*e.g.*, Mergner, 1971; Campbell, 1974), is morphologically simple compared to the comparable stage in the phyla most studied. The purpose of this paper is to determine if the planula stage of the Scyphozoa exhibits behavioral responses which can account for the distribution of its sedentary stage (the scyphistoma) in the field. The scyphistoma is usually found suspended upside-down from a hard surface in a shaded location (Wilson, 1952; Hardy, 1958; Fraser, 1962; Cargo and Schultz, 1966, 1967; personal observation), the position usually selected by marine larvae (*e.g.*, Crisp, 1974).

The response of the planulae of *Cyanea capillata* (Linneaus) to the direction of gravity and to light and their behavior on hard substrate of different texture or contour is examined in this paper. The effects that conditions associated with low levels of oxygen might have on their attachment behavior is considered, because scyphistomae are commonly found near the bottom of shallow estuaries (*e.g.*, Thiel, 1962) where these conditions must often be encountered by planulae. The settlement of *Cyanea* planulae is frequently accompanied by encystment (*e.g.*, MacMurrich, 1891; Hyde, 1894; Hargitt, 1902; Hargitt and Hargitt, 1910; Rees, 1957; Cargo and Schultz, 1967; Widersten, 1968; Cargo, 1975); the possible adaptive significance of this phenomenon is briefly discussed.

Materials and Methods

Planulae were obtained from gravid specimens of *C. capillata* (7 to 15 cm diameter) collected from Smith's Cove, Niantic, Connecticut, during the months of May and June, 1973 and 1974. Marshall (1960) provides a hydrographic description of this area.

General experimental conditions

Fresh, filtered sea water (salinity, 24‰; initial temperature, 15° C) was used in all experiments; these conditions correspond to those at the time the planulae were collected. The experiments were set up within three hours after obtaining the planulae and were run at room temperature (19–21° C) with one exception, noted below; the illumination was flourescent and constant.

Response to the direction of gravity

A 15-gallon aquarium, its sides screened with black construction paper, was used during the measurement of the response of planulae to the direction of gravity. Into this aquarium was placed an apparatus made of one-eighth inch diameter glass rods to which 2 cm^2 plastic coverslips were fastened with silicon-rubber cement; these rods were of different lengths and were attached to a lucite platform which rested on the aquarium frame. Thus, the coverslips were suspended at different heights above the aquarium bottom. Each height was replicated five times in a 5 × 5 Latin square, of which there were two: a lower set at heights of 5, 10, 20, 40, and 80 mm; and a higher set at heights of 100, 150, 200, 250, and 275 mm. Beginning at the time the planulae were added, observations were made at least twice within each 24 hour period for six days to determine the number of planulae swimming at different heights in a water column one cm wide, extending from the front to the back of the aquarium. When the planulae had attached, the numbers on the coverslips, the aquarium bottom and walls, and on the underside of the water surface (the hyponeuston) were recorded. The experiment was repeated with the positions of the low and high sets of coverslips reversed 180° relative to the aquarium.

Orientation of planulae at time of attachment and effect of surface texture

The surface upon which planulae attach, beneath or upon the uppermost surface of an object, and whether planulae distinguish between different surface textures were simultaneously determined. Planulae were added to a 20 cm diameter bowl in the bottom of which were arranged 16 plastic coverslips held 3 mm from the bottom by split-shot clipped to their corners. One half of the coverslips, on both the upper and the lower surfaces, was roughened with sandpaper (100 grit) to effect four treatments: R/R, R/S, S/R, S/S, where R represents rough, S represents smooth, and the letter to the left of the slash represents the upper surface. These treatments were arranged in a 4 × 4 Latin square. Two experiments of this design were run at the same time. The numbers of planulae attached to each of the coverslip surfaces, as well as the number which settled on the upper and the lower hemispheres of the split-shot, were recorded after they had attached.

Response of planulae to surface contour

The response to surface contour was determined by exposing planulae to 2 cm^2 plastic coverslips, the lower surfaces of which were scratched with a dissecting needle to produce shallow grooves in a grid-shaped pattern. The different coverslips were distinguished by the total length of grooves on their surface. There were two treatments: one with 10 linear cm and another with 49 linear cm of grooves; unmarked (0 cm) coverslips served as controls. The coverslips were arranged in a 3 × 3 Latin square, and were supported 3 mm above the bottom of an 11 cm diameter finger bowl.

Response of planulae to light

Shaded attachment sites. To establish whether planulae discriminate between shaded and nonshaded sites at the time of attachment, a 4 × 4 Latin square arrangement of supported coverslips were placed on the bottom of a 20 cm diameter bowl. One half of these coverslips were painted black on the upper surface, and one half of these coverslips were roughened on the lower surface. The four treatments were S/S, S/R, B/S, and B/R, where B represents the painted upper surface; the other letters have the same meaning as noted previously. A 30 cm tall cylinder of black paper, open at the top, was fit around the dish after the planulae had been added to increase the contrast in the level of illumination beneath the painted and the unpainted coverslips.

Direction of light. Planulae were exposed to four patterns of illumination to determine the effect that the direction of light might have upon their orientation at the time of attachment. The planulae in treatment i were put into 5 cm diameter (× 2.5 cm tall) covered stendor dishes and maintained in complete darkness. Black construction paper was fitted around the same sized dishes to make treatments ii, iii, and iv; a cylinder of black paper masked the dish sides in these treatments. Black paper covered the dish bottom (treatment ii) and the dish top (treatment iii) to allow light to enter from only one direction, from above and from below, respectively; in treatment iv, neither the top nor the bottom of the dishes was covered permitting illumination from both above and below the container. The dishes for treatments iii and iv were set on transparent glass plates to permit illumination from below. Ten replicates for each treatment were set up in random sequence with each replicate containing 20 planulae. The treatments were positioned randomly in two constant temperature cabinets: one cabinet was maintained without light (treatment i) and the other cabinet was evenly illuminated with directional light effected by black paper (treatments ii, iii, and iv). The temperature averaged 17.48 ± 0.147° C and 17.51 ± 0.170° C, respectively, for the two cabinets. The position of the attached planulae was determined on the 27th day.

Response of planulae to anaerobic conditions

Three air-tight containers to which planulae had been added were used to assess the effects which a lowered concentration of oxygen might have upon their attachment behavior: one contained deteriorating tissues from a moribund medusa and five supported coverslips; the other two contained fresh, filtered sea water and six supported coverslips, each. In one of the latter, air was bubbled, and in the other, nitrogen. After a period of two hours, the tubing was adjusted so that the water was no longer disturbed, but so that the volume above the water surface remained either pure air or pure nitrogen; an aperture in each container allowed introduced gas to escape. At the end of two days, the distribution of the planulae attached to upper and lower surfaces of the coverslips, to the bottom of the container beneath the coverslips, and to the hyponeuston above the coverslips was determined.

RESULTS

General observations

The mature planula is about 185 μ long, wider and blunt at the anterior end, biconcave in cross-section (Figure 1A), and yellow in color. Prior to settlement, the anterior-posterior axis becomes shortened and the cross-section becomes more circular. In this form, the planula appears to actively inspect (*sensu* Crisp, 1974) any surface with the anterior end by rotating counter-clockwise (viewed from the posterior pole) around its longer axis. If this surface is not suitable, the planula leaves it, swims for a variable distance, and then approaches the surface to inspect another site. Upon contact with a satisfactory site the planula becomes affixed and assumes a pyramidal shape (Figure 1B), which during three hours at 20° C becomes simultaneously reduced in height and increased in diameter (Figure 1C and 1D). The nipple at the apex disappears and a flange about 18 μ wide appears after six to ten hours (Figure 1E). This is the encysted planula from which, after a variable period of time, the polyp begins to emerge (Figure 1F). The encysted stage is firmly attached to the substrate, is an objective criterion for settlement, and is the stage enumerated in the experimental results which follow.

Analysis of the experimental results

Most of the data require transformation before analysis. Those data for which a log transformation is utilized are reported as antilogs (with 0.95 confidence limits). An angular transformation is used for proportional data (except for the observations on mortality); these results are reported as $P = 100[\Theta]^2$, (± s.d.). Bartlett's test for homogeneity of variances is nonsignificant (at least, $P > 0.10$) for all but one comparison which is mentioned in the appropriate place below.

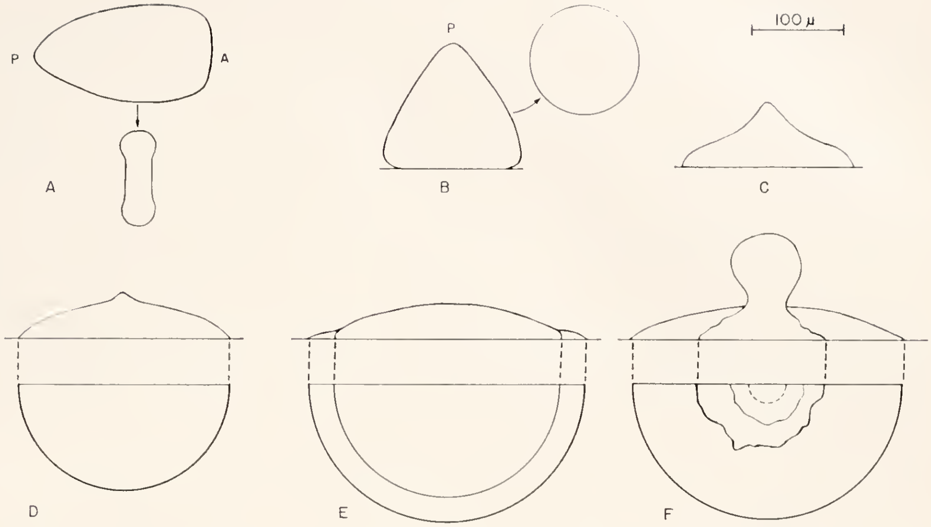

FIGURE 1. The early stages in the life history of *Cyanea capillata:* A, planula (view from above, upper diagram; cross-section, lower diagram); B, initial attachment (side view, left diagram; cross-section, right diagram); C, flattening of attached planula (side view); D and E, later stages and cyst formation (side view, upper diagram; view from above—one half of cyst shown, lower diagram); and F, excystment (views same as in D and E). A represents anterior; P, posterior (see text for details).

Tests of significance are based on the analysis of variance, model I. The row and column effects are nonsignificant in the experiments where a Latin square design is used.

Response to the direction of gravity

Planulae put into the aquarium accumulate on the bottom after only 30 minutes; the planulae move about slowly and concentrate in the lowest positions on the slate bottom until the 45th hour, filling in all grooves and depressions, which as a consequence appear as yellow lines and patches, respectively. Between 45 and 52 hours, their concentration in these surface irregularities are noticeably reduced as the planulae become more active and begin to swim less than five mm above the bottom. Planulae are conspicuous in the water column only after 69 hours at which time encystment begins on the coverslips suspended five mm above the bottom. At subsequent observations (between 72 and 144 hours), planulae remain in the water column. Throughout the latter period, concentrations of planulae are more or less stable up to a height of 8 to 12 cm; the maximum height attained by some planulae above this level (up to the surface of the water in the tank—28 cm) varies erratically until the last of these observations made on the sixth day. All planulae encyst by the ninth day.

The effect of this behavior on the position of attachment by planulae is shown by the distribution of their cysts on surfaces in the aquarium. In the two trials of this experiment, only 5.8% (1,518 cysts) and 17.3% (192 cysts) are attached to the aquarium bottom, respectively; the balance of the cysts are attached either to the suspended coverslips or to the hyponeuston, except for 0.6% (162 cysts) which are found on the aquarium walls in the first trial. The number of planulae which encyst on the coverslips—14,392 and 573, respectively—decreases linearly with distance from the bottom; this relationship is described by the following two equations: $\text{Log } \hat{Y} = \text{Log } 6306.2857 - \text{Log } 16.0844X$ and $\text{Log } \hat{Y} = \text{Log } 186.7542 - \text{Log } 7.6325X$, respectively. The regressions are significant ($P < 0.001$) for both sets of data, and the deviations from regression are nonsignificant ($P > 0.75$) for the former, but are significant ($P < 0.001$) for the latter, due to an unexplained heterogeneity.

The planulae apparently are initially geopositive and sometime between the 52nd and the 69th hour become geonegative prior to attachment; the planulae remain in contact with the bottom until between the 45th and 52nd hour. The linear gradient in density of attachment on suspended coverslips show that planulae attach most frequently close to, but not on, the bottom.

Orientation of planulae at time of attachment and effect of surface texture

The average number (with 0.95 confidence limits) and the number of planulae encysting on upper and lower surfaces of supported coverslips of different textures are shown in Table I; "coverslips (I)" and "coverslips (II)" show data from replicate experiments. Planulae encyst in greatest numbers beneath supported coverslips ($P < 0.001$) and upon a surface which is roughened ($0.025 < P < 0.05$).

The distribution of the planulae which encyst in positions other than on the coverslips was determined for those in one replicate experiment (I). These data for the planulae attaching to split-shot are shown in Table I: 84.6% ($n = 7474$) of the cysts are attached to the lower surface (hemisphere) of the shot ($P < 0.001$); but there is no difference ($P > 0.75$) in the number of cysts on the shot with respect to the rugosity of the coverslips that they support (columns, Table I). Note that

TABLE I

Orientation of planulae of Cyanea capillata *at time of attachment to coverslips and to split-shot and their response to surface texture.*

Orientation (surface of coverslip or split-shot)		Texture (coverslip)				Total	
		Rough		Smooth			
		Number attached	Mean (0.95 confidence limits)	Number attached	Mean (0.95 confidence limits)	Number attached	Mean (0.95 confidence limits)
Upper	Coverslips (I)	373	39 (22, 68)	196	20 (12, 35)	569	28 (19, 41)
	Coverslips (II)	621	73 (53, 101)	420	48 (33, 72)	1,041	59 (47, 76)
	Split-shot	662	68 (39, 119)	674	72 (44, 118)	1,336	70 (51, 97)
Lower	Coverslips (I)	8573	752 (350, 1618)	4614	465 (249, 867)	13,187	591 (377, 927)***
	Coverslips (II)	9036	975 (593, 1602)	6307	673 (394, 1151)	15,343	810 (580, 1131)***
	Split-shot	3562	416 (299, 579)	3912	435 (275, 687)	7,474	425 (332, 544)***
Total	Coverslips (I)	8946	171 (69, 426)*	4810	97 (38, 247)	13,756	
	Coverslips (II)	9657	267 (125, 569)*	6727	180 (83, 393)	16,384	
	Split-shot	4224	169 (95, 298) n.s.	4586	177 (99, 314)	8,810	

* $0.025 < P < 0.05$.
*** $P < 0.001$.
n.s. $P > 0.75$.

all of the shots possess the same surface characteristics; the data are indicated in separate columns to show the type of coverslip which the shot support. This difference between the outcome for split-shot and that for coverslip texture, above, strengthens the conclusion that roughened substrates attract greater numbers of planulae. The number of planulae attaching to the bottom, to the hyponeuston, or those not encysting at the time the counts were made (three days after the experiment began) total 352 (2.5%).

The basic design of the experiment to determine the response of planulae to "shaded attachment sites" is similar to that used in the experiment just described. The results of that experiment, in the present context are the same with regard to orientation at time of attachment, but differ in one respect in the response of planulae to texture. For example, 82.0% (n = 14,753) of the planulae attach to the lower surface of coverslips, and 88.9% (n = 13,112) of these encyst beneath coverslips with a rough lower surface. However, of the 18% (3223) of the planulae which settle on the upper surface, an average of 346 (251, 477) planulae attach to each coverslip which had the upper surface painted, compared to an average of 34 (24, 47) planulae attaching to the unpainted (smooth) upper surface of coverslips; this is a 10-fold difference. In contrast, the ratio of the average number of planulae encysting on the rough upper surface to those attaching to smooth (upper surface) coverslips shown at the top of Table I for this experiment are 1.95 (39:20) and 1.52 (73:48), respectively, for the two trials. This suggests that planulae respond to the fine texture (pigment granules) of the painted surface more strongly than to a course roughness (sandpapered surface).

Response of planulae to surface contour

There is a positive linear relationship between the length of the grooves scratched on the lower surface of coverslips and the number of planulae which attach to them during a 24 hour period. On unetched (control) coverslips, 238 ± 48 planulae encyst; on coverslips with 10 linear cm of scratched surface, 554 ± 44 planulae attach; and on those with 49 cm of grooves, 1180 ± 103 planulae settle.

Response of planulae to light

Shaded attachment sites. The distribution of planulae encysting after nine hours beneath opaque (painted) coverslips and beneath transparent coverslips does not differ ($P > 0.75$). The average number (and 0.95 confidence limits) on the undersurface of each opaque coverslip was 512 (198, 1324) and underneath each transparent coverslip, 444 (114, 1719). No free-swimming planulae remained at the time counts were made.

Direction of light. The effect of different patterns of light on the orientation at time of attachment and the mortality of planulae is shown in Table II. The angular transformation of the data reduces, but does not eliminate, the heterogeneity of variances. An approximate test for these data show that the proportion of planulae which encyst upside-down (on the hyponeuston) is highest ($P < 0.001$) if the light is projected from beneath their container. An *a posteriori* test shows no difference in the proportion which attach upside-down among the remaining three treatments. These results suggest that the planulae are photo-negative at time of attachment, settling with greatest frequency on a surface opposite the light source, but since the absolute differences among the remaining treatments are slight this response to light appears to be weak relative to their orientation to direction of gravity.

Mortality of planulae is greatest ($P < 0.001$) for those maintained in total darkness ("none", in Table II); there is no difference in mortality among treatments which receive light ($0.10 < P < 0.25$).

Fifteen planulae (1.97%) were unattached when the experiment was terminated on the 27th day; the distribution of these among the treatments can be determined from the data provided in Table II. Neither their attachment nor their death would change the outcome above.

Response of planulae to anaerobic conditions

The orientation at the time of attachment by planulae both on coverslips and in different positions in their container under anaerobic conditions is shown in Table III (no planulae attached to the walls of their container). The initial number (not known) of planulae used in each treatment definitely was more similar than shown in Table III. The differences in the number of planulae among the three treatments possibly are, on the one hand, due to an increase in planulae in the treatment to which organic matter was added because of completed embryogeny from blastulae associated with the gonadal tissue; and on the other, due to higher mortality in the treatment with nitrogen.

Both sets of data (coverslips and container) indicate that relatively few planulae attach rightside-up when exposed to air, and that proportionately more planulae attach rightside-up in the absence of oxygen (nitrogen) than in the treatment with

TABLE II

Effect of different illumination on the orientation at time of attachment and the mortality of planulae of Cyanea capillata.

Position of light source (number of planulae)	Attachment			Mortality	
	Site	Number attached	Percent surviving planulae attached upside-down (±s.d.)	Number	Percent (±s.d.)
None (200)	Hyponeuston Dish bottom	129 19	91.0 ± 6.07	52	26.0 ± 9.37***
Above culture (200)	Hyponeuston Dish bottom	154 18	92.9 ± 3.46	18	9.0 ± 6.15
Below culture (200)	Hyponeuston Dish bottom	171 1	99.9 ± 0.56***	26	13.0 ± 8.23
Above and below culture (160)†	Hyponeuston Dish bottom	127 15	92.2 ± 3.89	15	8.0 ± 6.75

† Two replicates with 20 planulae each were lost.
*** $P < 0.001$.

TABLE III

The orientation at time of attachment by planulae of Cyanea capillata *in response to anaerobic conditions.*

Treatment	Site of attachment	Number of planulae attached	Percent of planulae attached rightside-up (± s.d.)
Air (control)	Coverslip		
	Upper	51	6.3 ± 0.71**
	Lower	772	
	Container		
	Hyponeuston	404	4.6 ± 9.63***
	Bottom	11	
Organic material†	Coverslip		
	Upper	133	8.1 ± 0.55
	Lower	1888	
	Container		
	Hyponeuston	123	59.8 ± 11.29
	Bottom	168	
Nitrogen	Coverslip		
	Upper	148	30.3 ± 1.97***
	Lower	331	
	Container		
	Hyponeuston	17	77.1 ± 14.40*
	Bottom	72	

† Organic material represents pieces of oral folds, gonadal tissue, and tentacles from a medusa.
* $0.025 < P < 0.05$; ** $0.001 < P < 0.005$; *** $P < 0.001$.

organic material added; in the latter, the oxygen content could be expected to be at an intermediate level. An *a posteriori* comparison shows that the difference in the proportion rightside-up on coverslips between air and organic material is nonsignificant.

Three hours after this experiment was set up, many planulae were observed to be active and swimming throughout the water volume in the treatment to which organic material was added; very few planulae were seen in the treatment exposed to nitrogen. No planulae were observed in the control which means that they must have been on the bottom (the refraction at the bottom of the glass dishes prevented observation from the side, and the covers were translucent). The possible significance both for this difference in activity, and for the results showing orientation of planulae at time of attachment in Table III, are made more clear by a fortuitous observation during the first experiment ("response to the direction of gravity") described in this section.

In the two trials of the first experiment, few planulae attach rightside-up on the bottom of the aquarium. In contrast, during another trial (not reported) intended to yield identical data, the results are different: 61.6% (n = 19,296) of the planulae encyst rightside-up; and prior to encystment they swim throughout the water column without an initial settling, *en masse,* to the bottom. However, in setting up this trial the expedient was taken neither to filter nor to aerate the water used before adding the planulae. Examination of this water to determine the vertical distribution of the planulae showed it to contain a dense population of ciliates, in excess of 10^5 cells/ml (estimate made later). Such large populations of protozoa could alter the dissolved gas content of the medium.

Thus, planulae might become more active and swim away from the bottom under conditions associated with low oxygen; under these conditions in the laboratory, attachment is most frequently rightside-up.

These laboratory experiments are not considered to be a substitute either for experimentation or for observation in the field; however, these data do provide the basis for a reasonable hypothesis which can focus field investigation.

Discussion

These data suggest the sequence of events which may occur in the field during the early larval period of *C. capillata* (see Figure 2). The planulae, upon leaving the oral folds of the parent medusa, descend quickly to the bottom (Figure 2A) where their continuing movements take them to the lowest level within the area of their initial settling (Figure 2B). The consequences of this are two-fold: first, the planulae are most likely to encounter anaerobic conditions at the water-sediment interface (see below) which stimulates them to swim upwards (Figure 2C); and second, swimming upwards from the lowest position increases the probability of contact with the undersurface of an object. The latter possibly is enhanced by a weak photonegative response (Figure 2D) which inhibits the planulae from swim-

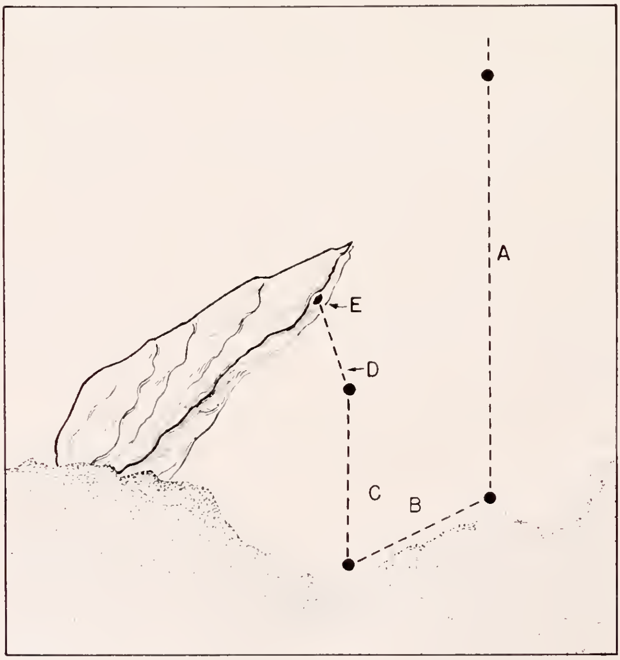

FIGURE 2. The pattern of behavior shown by the planula of *Cyanea capillata* in the laboratory extrapolated to conditions in the field (see text for details).

ming around the margin of an object. Attachment to the substrate, by encystment. follows the selection of an appropriately rough site (Figure 2E). This sequence leads to the attachment by planulae upside-down.

The attachment upside-down by planulae of *Cyanea* has been noted by others. Perez (1920–21) observed the attachment of *Cyanea* planulae to the undersurface of pieces of mussel shell placed with them in culture dishes, and Phillips (1972) mentions similar observations; I have obtained the same results on shells of *Mytilus*, *Modiolus*, and *Mercenaria*. However, a number of authors remark upon the settlement of scyphozoan planulae rightside-up on the bottom of their containers (Sars, 1841; Hargitt and Hargitt, 1910; Perez, 1920–21), and others imply that this is the usual orientation at the time of attachment (Agassiz, 1862; MacMurrich, 1891; Cargo, 1975). This study shows that significantly more planulae attach upside-down in fresh sea water than in the opposite position; this is consistent with field observations on the position occupied by scyphistomae.

The polyps of *Aurelia* are most frequently found attached upside-down (Wilson, 1952; Hardy, 1958; Fraser, 1962), and the photographs in Buchsbaum (1972) show this orientation, as does the picture of strobilating polyps of *C. capillata* [?] in Jägersten (1960). I have seen scyphistomae (sp.?) attached upside-down on rocks collected from the northwest Pacific. This is also the usual orientation for the scyphistoma of *Chrysaora quinquecirrha* on oyster shells in Chesapeake Bay (Cargo and Schultz, 1966, 1967; Cones and Haven, 1969). In addition, Cargo and Schultz (1967) report field experiments which show that more planulae of *C. quinquecirrha* attach to the lower surface of suspended glass plates than to the upper surface, but regard their data to be insufficient to reach a definite conclusion.

The orientation to, and the selection of, specific sites by marine larvae represents their final response to a series of environmental cues. Initially, most larvae in the higher phyla are negatively geotactic and/or positively phototactic upon release; reversal of these taxes, associated with age and/or developmental stage (Reese, 1964; Newell, 1970; Meadows and Campbell, 1972; Crisp, 1974), occurs prior to attachment. This pattern of response to light (and possibly also to gravity) is apparently true for madreporarian planulae (*e.g.,* Yonge, 1963, 1968).

With respect to light, Williams (1965) shows that the planulae of *Clava squamata* (Hydrozoa) are initially photopositive, becoming photonegative with age, especially if in contact with suitable algal substrate; at time of settlement, the planulae of *Hydractinia echinata* (Hydrozoa) are photopositive (Teitelbaum, 1966). On the other hand, at least the early planulae of *Cyanea* appear to be indifferent to light; many hours of observation with both flourescent and incandescent light at different intensities and from different angles give no indication that light influences their early behavior. Cargo and Schultz (1966) indicate a lack of photosensitivity in the planulae of *C. quinquecirrha*, and the planulae of *Eunicella stricta* (Octocorallia) do not respond to light either (Theodor, 1967). However, *Cyanea* planulae may be photonegative just prior to attachment, although Phillips (1972) remarks that the settlement of planulae of *Cyanea* on shell surfaces appears not to be affected by light; further work is required on the photic response, for this is affected not only by light intensity, but also by salinity and temperature (*e.g.,* Meadows and Campbell, 1972) and neither these parameters, nor others (see Crisp, 1974), are explored in the present study. Some illumination does seem to be necessary for optimal survival during the planula stage of *Cyanea,* perhaps by increasing attachment rate and thereby reducing the length of the free-swimming stage; madreporarian planulae attach more slowly in the absence of light (see Yonge, 1963, 1968). In the experiments reported, here, the response to light was weak and almost obscured by the response to direction of gravity.

The early planula of *C. capillata* are geopositive; Phillips (1972) notes the initial movement of *Cyanea* planulae to the bottom of culture dishes where they swim next to the substrate. The planula of *E. stricta* are possibly geopositive, also; their descent to the bottom is passive, however, and Theodor (1967) does not regard this to be a behavioral response. Whether the initial settlement of *Cyanea* planulae is an active response was not determined. Even if this is solely due to the effect of gravity, settlement to the bottom might be considered an active response, for *Cyanea* planulae are capable of swimming to maintain their position in the water column if the water contains large quantities of organic matter, living or dead. Perez (1920–21) records the activity of *Cyanea* planulae in his collecting buckets containing medusae, and this author has noted the same in those buckets left to stand unattended (medusae readily shed bits of tentacles and other tissues from the oral surface under these conditions).

It appears that a reversal of response to the direction of gravity is stimulated by anaerobic conditions; in effect, the planula becomes geonegative when exposed to a lowered oxygen concentration or associated factors. Additional work is required to determine whether this really is a response to a lowered concentration of oxygen or a response to an increase of carbon dioxide. The fact that planulae are more active in containers with organic matter than in those in which oxygen is replaced with nitrogen suggests that the latter is possible.

The planulae cultured in fresh, filtered sea water may create the stimulus for activity through their own metabolism, since they become densely concentrated in local patches at the bottoms of their containers. Populations of protozoa alter the oxygen tension (Fox, 1921) or carbon dioxide concentration (Jennings, 1906) in their immediate environment to which they respond by adjusting their distribution in this self-induced gradient. The ciliate-like planula might be expected to react to these conditions in a similar way.

Because the stimulus for increased activity of the planulae may be associated with the concentration of dissolved gases, it is significant that scyphozoan polyps are most frequently found in the field in areas of calm water (*e.g.,* Thiel, 1962) on hard substrate in close proximity to where sediments are fine and thus most conducive to anaerobic conditions at the water-sediment interface (*e.g.,* Hayes, 1964; Fenchel, 1969); under these conditions a gradient in dissolved gases (and other chemical characteristics) which extends a few millimeters above the sediment surface can become established (*e.g.,* Odum, 1971, pp. 343–344).

Planulae cultured in containers having a high concentration of organic matter attach rightside-up; this orientation at time of attachment is opposite of that occurring in most of the experiments. The data do not provide a direct answer for this paradox. This difference may be related to either or both the rate of change in the concentration of dissolved gases or to the extent of this change. Under extreme anaerobic conditions the planulae may not be capable of sustained activity, they sink, and attach rightside-up where they land. It was noted, above, that the planulae are not particularly active in the culture exposed to nitrogen; more attach rightside-up in this treatment than in either that which contains organic material or in the control. The possibility that mortality is highest in this treatment with nitrogen is also mentioned and is consistent with this interpretation. This suggests that attachment rightside-up may be, at least in part, a laboratory artifact which occurs under more extensive anaerobic conditions than would be expected in the field.

Response of planulae to physical characteristics of the substrate has been reported for Anthozoa (Yonge, 1963, 1968; Theodor, 1967) and for Hydrozoa (Williams, 1965; Teitelbaum, 1966) and is a well-recognized phenomenon among marine larvae in higher phyla (Reese, 1964; Newell, 1970; Meadows and Campbell, 1972; Crisp, 1974). Williams (1965) shows that planulae of *C. squamata* respond to substrate surface in two ways: first, they selectively settle in small depressions in a hard surface of uniform texture; and secondly, they are sensitive to surface roughness as measured by their rates of locomotion on different artificially roughened substrates. The first response is similar to that shown by the planulae of *Cyanea* which select grooves and depressions on the bottom of their container, although this is not a pre-attachment phenomenon in *Cyanea* as it is for *Clava*. The second response may be shown by the different numbers of *Cyanea* planulae encysting on the upper surface of smooth, roughened, and painted coverslips which suggests the capacity to discriminate among these three grades of roughness, but the rates of movement of the planulae resulting in this distribution of cysts was not measured. The possibility that this difference is due to an albedo response is lessened if it is recalled that planulae did not attach to the black slate bottom of the aquarium under identical illumination; however, response to a chemical difference on the painted coverslips cannot be ruled out. The response to substrate contour at time of attachment is shown in the relative distribution of planulae attaching to coverslips with grooves scratched on their lower surface; this may be the rugophilic response similar to that described by Crisp and Barnes (1954) for cyprid larvae, and since shown to be common among many marine larvae (Crisp, 1974). Likewise, planulae were observed to concentrate in the grooves made by pliers on split-shot when fastening them to coverslips. These laboratory data for *Cyanea* agree with the field observations of Cones and Haven (1969) who say that polyps of *C. quinquecirrha* are most numerous in depressions and along cracks in the surface of oyster shells.

The frequency with which planulae attach and metamorphose on the hyponeuston (Sars, 1841; Hargitt and Hargitt, 1910; personal observation), a smooth surface, may be another artifact associated with laboratory culture. The same phenomenon occurs with some sponge larvae and is similarly interpreted (see references in Fell, 1974).

Assuming, with appropriate reservation, that the events observed in the laboratory can be extrapolated to the field, these data suggest that under natural conditions attachment by planulae may be relatively rapid, being initiated not long after they encounter the conditions which occur at the water-sediment interface. The sometimes long and variable length of larval life observed during these experiments may be yet another laboratory artifact. The larvae of higher invertebrates can delay attachment in the absence of appropriate stimuli (*e.g.,* Newell, 1970; Crisp, 1974), and the same phenomenon has been observed by Phillips (1972) for *Cyanea* and by Cazaux (1958, 1961) for *H. echinata* (Hydrozoa).

It seems reasonable that the motile planula stage of *Cyanea* is of short duration. Long larval life is selectively advantageous for sedentary species in which this is a dispersal stage (*e.g.,* Thorson, 1950, 1961; Scheltema, 1967, 1971). In the Scyphozoa, unlike the situation in higher phyla with sedentary adults, the adult (pelagic medusa) is best suited for dispersal. The small, weakly swimming and primarily benthic planula might not be expected to serve this function effectively. Mackie (1974) questions the role of dispersal traditionally accorded the medusa, and emphasizes the selective advantage of maintaining a circumscribed distribution in shallow water where the sedentary phase of the cnidarian life history may be successfully completed. The rapid settlement to the bottom and subsequent attachment by the planula of *Cyanea* would fulfill this requirement. The probable limited movement of the planula on the bottom would involve solely the selection of a site for attachment, in a location presumably advantageous for the survival of the polyp.

Advantages for the polyp are uncertain. It might be that attachment on the underside of an object decreases the unfavorable consequences of sedimentation and overgrowth by other organisms (Cargo and Schultz, 1966). Also, in this position above the substrate the exposure to prolonged anaerobic conditions is reduced, and the array of potential food organisms possibly is increased. In addition, the process of strobilation and subsequent release of ephyrae may be more efficient from a pendant attitude.

The term *encystment,* applying to the planulae at time of attachment, implies a protective function for this stage between the free-swimming larva and the sedentary polyp. Preliminary observations suggest this is a possibility, but convincing data are not yet available and the observations of others conflict. MacMurrich (1891) reports almost universal encystment by planulae of *C. arctica* [= *C. capillata*], and personal observations, as well as those of Cargo and Schultz (1967) and Cargo (1975), indicate that encystment is the rule in *C. capillata;* similarly, Rees (1957) and Widersten (1968) report that encystment is a normal ontogenetic stage in the life history of *C. lamarcki* and *C. palmstruchi*, respectively. On the other hand, Hargitt (1902) and Hargitt and Hargitt (1910) suggest that encystment is facultative in *C. arctica* [= *C. capillata*] and is an adaptation to unfavorable conditions; the data of Hyde (1894) can be interpreted to support this conclusion. Resistance to summer temperatures by these cysts is implicit in Cargo (1975). The failure to mention encystment by *C. capillata* suggests that it does not occur in the material observed by Agassiz (1862), Perez (1920–21), or Widersten (1968); Sars (1841) implies that the cyst is an attachment disc. This observation by Sars (1841) suggests the possibility that the term *encystment*, applied to this stage in much of the literature, is inappropriate; Mergner (1971, p. 20) describes the formation of periderm-surrounded discs after the attachment and flattening by hydrozoan planulae, similar to observations of *Cyanea* made by this author and presumably others.

Russell (1970) notes that planula cysts of *Cyanea* have never been found in nature. Since the scyphistomae are capable of limited locomotion (Gilchrist, 1937; Chapman, 1966, 1968; Blanquet, 1972), the discovery of planula cysts of *Cyanea* in the field would not only provide clear evidence for the choice of attachment site (because they are fixed immovably to the substrate), but may also contribute to a better understanding of the phenomenon of encystment in this species.

Neither the effects of water movement nor the chemical influence of the substrate surface on attachment behavior of *Cyanea* planulae was studied. Either one or the other, or both, may affect the behavior of some larvae in the higher phyla (*e.g.*, Reese, 1964; Newell, 1970; Meadows and Campbell, 1972; Crisp, 1974), and similarly that of cnidarian planulae (Cazaux, 1958; 1961; Williams, 1965; Teitelbaum, 1966; Nishihira, 1965, 1967, 1968a, b, c; Theodor, 1967; Müller, 1969).

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SUMMARY

The behavior of the planula of *Cyanea capillata* (Linneaus) is studied in the laboratory to determine if it responds to environmental cues in a way which may account for the distribution of its sedentary scyphistoma stage in the field.

The planulae react to gravity, water chemistry, surface texture, and possibly to light. They exhibit an initial geopositive response in well-aerated sea water, but become active and geonegative under conditions associated with local depletion of oxygen. Their attachment follows, and is usually upside-down on a horizontal surface. They attach with greatest frequency on substrates that are rough, and give evidence of discriminating among surfaces of different rugosity. The planulae possibly show a weak photonegative response at the time of attachment; confirmation of this photic response requires further work. These sequential responses shown in the laboratory result in an orientation at the time of attachment which is in agreement with the position usually occupied by the scyphistoma in the field.

The behavior of the planulae of *C. capillata* demonstrates a pattern of responses which may confirm, and extend to the Scyphozoa, the conclusion of others that a morphologically simple larva as found in the Cnidaria has the capacity to non-randomly select specific substrate upon which to settle, a phenomenon well-documented for the more complex larval stages found in the higher Metazoa.

The attachment by planulae to the hyponeuston, their attachment rightside-up under anaerobic conditions in the laboratory, and the sometimes long duration of the motile phase of larval life are thought to be artifacts of laboratory confinement.

The adaptive significance of encystment at time of attachment is unresolved.

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THE REPRODUCTION OF *HALICLONA LOOSANOFFI* AND ITS APPARENT RELATIONSHIP TO WATER TEMPERATURE[1]

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There have been few detailed studies on the reproductive periods of sponges and very few on the reproduction of particular species within different parts of their geographical ranges (Fell, 1974a and 1976). Usually studies of sponge reproduction have involved either examination of tissue samples taken at regular intervals throughout the year or observation of larval settlement on experimental surfaces. Rarely has an attempt been made to compare the results from these two methods of study (Bergquist and Sinclair, 1973).

Water temperature has been frequently assumed to be a major environmental factor regulating the reproduction of sponges. Although such regulation would not be unexpected, it has been documented in few cases (Storr, 1964; Wells, Wells and Gray, 1964; Simpson, 1968). Consequently more extensive information concerning the relationship between water temperature and reproductive activity would be of interest.

The present study defines the reproductive period of *Haliclona loosanoffi* in the Mystic Estuary, Connecticut using three different approaches: 1) examination of tissue samples from adult specimens, 2) observation of larval release from freshly collected specimens in the laboratory, and 3) study of larval settlement on mollusc shells suspended in the estuary. These studies confirm preliminary results (Fell, 1974b) which indicated that the reproductive period of *Haliclona loosanoffi* is much earlier at Mystic than at Milford, Connecticut (Hartman, 1958) and occurs at about the same time as in more southern regions (Wells *et al*, 1964; Marsh, 1970). Because of this unexpected situation, it seemed of interest to determine whether the reproductive period could be related to water temperature. The evidence is consistent with the hypothesis that water temperature is a major factor regulating reproduction in this species.

Since previous studies on the reproduction of *Haliclona loosanoffi* were almost exclusively concerned with larval settlement (Hartman, 1958; Wells *et al*, 1964), little was known about the type of sexuality, gametogenesis and embryonic development. Information on these subjects was obtained in this study by the examination of tissue samples. In addition, some new observations concerning the selection of settling surfaces by the larvae of this species were provided by the larval settlement studies.

MATERIALS AND METHODS

Specimens of *Haliclona loosanoffi* were collected from the lower Mystic Estuary, Connecticut at regular intervals during five years (1969–1971, 1973, and 1974).

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In many cases the specimens were fixed immediately in Bouin's solution in sea water, but in some cases they were studied in the laboratory before they were fixed (see below). The fixed specimens were cut into pieces and examined under a dissecting microscope for the presence of large oocytes and embryos. Small samples of the specimens were then embedded in paraffin and sectioned serially at 10 μ. The mounted, deparaffinized sections were stained with hematoxylin and eosin. Usually a minimum of 5 spaced serial sections were scanned at a magnification of 1000 × to determine the presence or absence of small oocytes or spermatic cysts, and additional sections were examined at lower magnifications. In all more than 150 specimens were studied histologically.

During one summer (1974) specimens of *Haliclona loosanoffi,* collected at weekly intervals, were brought back to the laboratory and placed in finger bowls containing sea water for 6 to 12 hrs to see if they would release larvae. From 2 to 14 (usually 5 or more) specimens were examined on each date, and the sea water in which they were placed was filtered, freshly collected sea water from the Mystic Estuary. Only intact specimens, which were resting on sandy bottom, were used. On one occasion when large numbers of larvae were released by the sponges, observations on larval behavior and settlement were made in the laboratory. The sponges were carefully removed from the bowls, and the larvae were kept in the original sea water into which they were released.

In 1971 larval settlement was studied by suspending mollusc shells in the Mystic Estuary at regular intervals. Freshly shucked shells of the clam, *Mercenaria mercenaria,* were thoroughly cleaned in fresh water and completely dried. A small hole was drilled near the center of each shell, and the shells were strung in groups of 5 on nylon cord with the concave (inner) surface down and tygon tubing spacers (1.5 inches long) between successive shells. From 1 May to 16 August ten shells were placed in the estuary below the level of tidal exposure every three weeks. Each time a new set of shells was suspended in the estuary, the preceding set was removed. The shells were examined for the presence of sponges under a dissecting microscope as soon as they were brought back to the laboratory, and the identification of each small sponge was checked by preparing a wet mount of it and examining the preparation at a magnification of 430 ×. Skeletal characteristics were considered diagnostic.

The size index of many specimens was determined by multiplying the length × width × height. When specimens were of irregular shape, they were cut into a number of more or less regularly shaped pieces and the size indices of the individual pieces were summed.

RESULTS

General description of reproduction

Haliclona loosanoffi apparently is gonochoric, there being a separation of the sexes. While this is true, what appear to be abortive small oocytes are found in many male specimens during the latter part of the reproductive period. The sex ratio is approximately 1 : 1. Of 71 specimens examined during the period when all specimens were reproductive (last week of May through the first week of July), 34 were males and 37 were females.

Gametogenesis and embryonic development in *Haliclona loosanoffi* are virtually identical to these processes in *Haliclona ecbasis* which has been studied in detail (Fell, 1969, 1970). The sperm develop within spermatic cysts which are scattered throughout the endosome. Each cyst is made up of one or more clusters of spermatogenic cells surrounded by a layer of pinacocytes. All of the cells of a cluster are at the same stage of development, but different clusters within the same cyst may be at different stages. The sperm have a spherical head approximately 1 μ in diameter and a tail about 10 μ in length. All of the sperm in a cluster are oriented in the same direction. Frequently all stages of spermatogenesis occur simultaneously in the same specimen.

The oocytes are individually distributed throughout the endosome. At an early stage of growth an oocyte becomes surrounded by nurse cells which it progressively engulfs. The nucleus of the engulfed nurse cells disappears but the cytoplasm appears to remain unchanged. Consequently the larger oocytes and early embryos are filled with a large number of nurse cells, and little oocyte (or blastomere) cytoplasm can be discerned. After the engulfment of nurse cells is complete, the oocyte becomes enclosed within a thin follicular envelope consisting of flattened pinacocytes.

Distinct blastomeres usually are not detectable during the initial cleavage stages, presumably because of the close packing of the cells. The early embryos are therefore difficult to distinguish from the larger oocytes at low magnification. As cleavage proceeds the engulfed nurse cells are gradually fragmented and incorporated into the cytoplasm of the blastomeres. When this process is complete, the embryo develops into a solid, flagellated larva (parenchymula). Spicules appear at the periphery of the embryo during late cleavage, but as the larva develops they become situated internally near the posterior pole. Mature larvae leave the parent sponge by way of the excurrent water system.

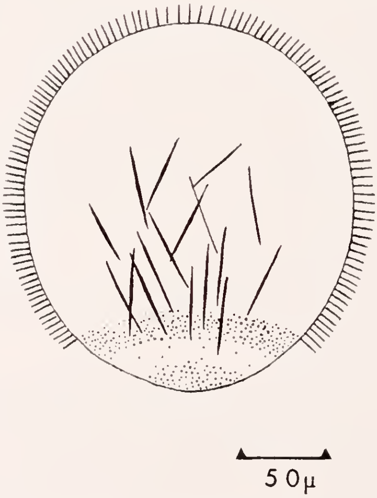

FIGURE 1. Free-swimming parenchymula larva of *Haliclona loosanoffi.*

It is not uncommon for all stages of oogenesis and embryonic development to be present in a single specimen at the same time.

The free-swimming larva of *Haliclona loosanoffi* measures approximately 188 × 172 μ. Most of the larva is light beige and a golden-brown ring encircles the posterior pole. In many cases an area of brown pigment occurs in the center of the ring. This may be sufficiently large in some larvae that the posterior pole is almost completely pigmented. Short flagella cover the entire surface of the larva except for the posterior pole. The larval spicules (oxeas) are about 50–60 μ in length (Fig. 1).

Reproductive period

The earliest that active specimens of *Haliclona loosanoffi* have been found in the Mystic Estuary is late May or early June. Already at this time all of the specimens contain large numbers of gametes and/or embryos. This situation persists through the third week of June, and then the percentage of specimens with large numbers of reproductive elements steadily declines reaching a low level by the end of July (Fig. 2A). As would be expected, female specimens contain primarily small oocytes at the beginning of the reproductive period. Then the small oocytes become less numerous and the number of embryos steadily increases. Finally, during the period when mature larvae are released (see below), the embryos progressively decrease in abundance (Table I). Growing oocytes and mature larvae never constitute a large proportion of the reproductive elements.

During the first part of the reproductive period male specimens possess a large number of large spermatic cysts. Some of the larger cysts measure about 100 × 50 μ. Then both the number and size of the cysts decline. Beginning about the fourth week of June, the larger cysts are only about half the size of those found earlier in the season. These smaller cysts generally are composed of a single cluster of spermatogenic cells. By the second week of June, some of the cysts containing mature sperm appear largely empty, suggesting that most of the sperm have been released.

Although actual reproduction (as judged by the presence of embryos and larvae in the specimens) ends by late July or early August, some specimens with spermatic cysts or small oocytes may be found until at least the end of September.

During the early part of the reproductive season there is a considerable variation in the size of the specimens of *Haliclona loosanoffi*, depending in part upon the number of gemmules which contributed to their development. Twenty four specimens collected in late May and June had size indices (length × width × height) ranging from 0.024 to 16.7 cm^3 (mean *ca.* 2.0 cm^3). The density of reproductive elements was high in all of these sponges, indicating that within broad limits size is not an important factor affecting reproductive activity.

Flagellated parenchymula larvae are found in histological sections of specimens collected from the second week of June through the first week in August (Fig. 2C). The release of larvae by freshly collected specimens in the laboratory (Fig. 2B) and larval settlement on experimental surfaces (Fig. 2A) both indicate that larvae are released throughout this period. The greatest larval settlement seems to occur in mid-July.

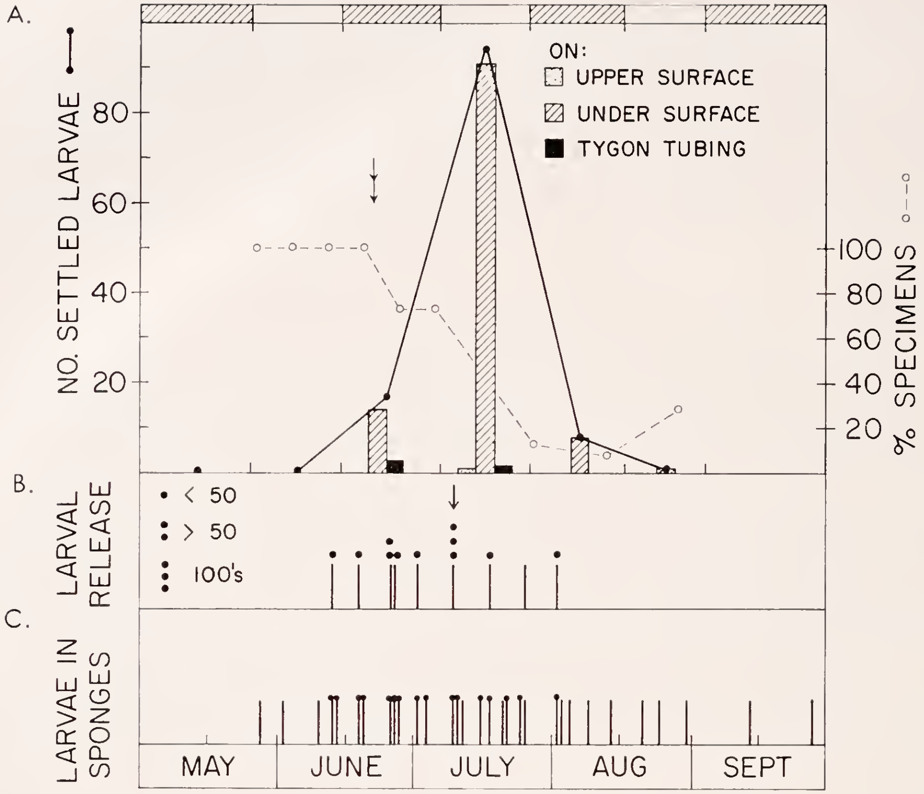

FIGURE 2. Reproduction of *Haliclona loosanoffi* in the Mystic Estuary, Connecticut. *A.* Reproductive activity and larval settlement: Right—percentages of specimens (both male and female) with large numbers of reproductive elements calculated on a weekly basis (11 to 16 specimens were examined for each point, excepting the first and last for which 5 and 7 specimens were examined, respectively). Left—the number of larvae settled on 10 *Mercenaria* shells per 3-week interval. The shells were strung with the concave surface down and tygon tubing spacers between them. The pattern of settlement on available surfaces is indicated. The double arrow marks the earliest date that large numbers of small postlarvae were observed on eel grass in the same area. *B.* The release of larvae by freshly collected specimens of *Haliclona loosanoffi* in the laboratory. Vertical lines show when observations were made, and dots indicate the greatest number of larvae released per specimen. The arrow marks the only date that a larva of this sponge was found in plankton samples. *C.* The occurrence of flagellated larvae in specimens of *Haliclona loosanoffi* collected at different times during the late spring and summer. Vertical lines represent collections of specimens made over a 5-year period; when dotted, flagellated larvae were found.

Eel grass (*Zostera marina*) in the Mystic Estuary provides a very large surface area for larval settlement, and the greatest number of larvae apparently settle on this substrate. Heavy settlement of *Haliclona* larvae on the eel grass was first observed near the end of June. The postlarvae grow rapidly and by late summer may achieve a size index of about 15 to 45 cm^3.

TABLE I

Numbers of reproductive elements per cm² histological section (10 μ thick) and the relative abundance of different kinds of reproductive elements in female specimens of Haliclona loosanoffi *during different periods within the reproductive season. Means (in italics) and ranges (in parentheses) are given. (Large oocytes are defined as those containing engulfed nurse cells; embryos are defined as cleavage stages and developing larvae.)*

Period		Number of specimens examined	Number of reproductive elements/cm²	Percent total number of reproductive elements			
Month	Week			Small oocytes	Large oocytes	Embryos	Flagellated larvae
May	4	3	*432* (265–540)	*97* (94–100)	*2* (0–3)	*1* (0–3)	*0*
June	1	5	*570* (392–758)	*74* (41–100)	*3* (0–5)	*23* (0–54)	*0*
June	2	5	*572* (327–1064)	*48* (20–83)	*3* (1–7)	*48* (10–75)	*1* (0–3)
June	3	8	*388* (175–771)	*40* (20–79)	*2* (0–3)	*57* (21–75)	*1* (0–4)
June	4	7	*272* (82–934)	*44* (9–98)	*1* (0–3)	*52* (2–85)	*2* (0–4)
July	1	6	*141* (59–302)	*62* (29–90)	*1* (0–2)	*35* (10–65)	*2* (0–5)
July	4	8	*59* (4–327)	*90* (20–100)	*0.1* (0–1)	*9.8* (0–78)	*0.1* (0–1)

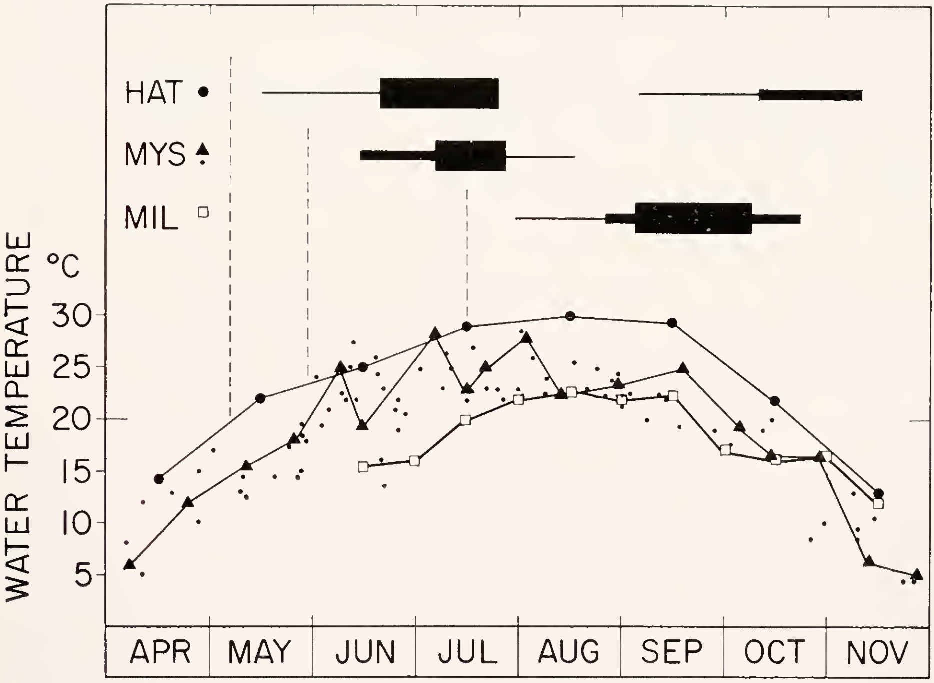

FIGURE 3. Water temperature and periods of larval settlement of *Haliclona loosanoffi* at Hatteras Harbor, North Carolina, the Mystic Estuary, Connecticut and Milford Harbor, Connecticut. The large symbols connected by lines show the surface temperatures during the years that larval settlement was studied; the dots indicate the water temperatures in the Mystic Estuary during other years. The relative numbers of larvae settling on experimental surfaces during each interval of observation are indicated by the thickness of the bars. Information from Hatteras Harbor is taken from Wells, *et al* (1964); and that from Milford Harbor is taken from Hartman (1958). Larval settlement at Milford during 1947 is shown. Vertical broken lines show the approximate time that water temperature reaches 20°C at each location.

TABLE II

Settlement of the larvae of Haliclona loosanoffi *on the shells of* Mytilus edulis *in the laboratory with natural photoperiod. (The development of settled larvae on the outer shell surface appeared to be somewhat superior to that of larvae on the inner shell surface. Larvae also settled in large numbers on the sides of the glass containers.)*

Shell surface	Number of settled larvae		
	When facing down	When facing up	Ratio
Outer surface	249	32	7.8
Inner surface	175	38	4.6
Both surfaces	424	70	6.0

Although free-swimming larvae of *Haliclona loosanoffi* apparently are present in the Mystic Estuary for about one and a half months, such a larva was found in plankton tows on only one occasion (in early July). On the other hand, the larvae of a species of *Halichondria* were regularly taken in plankton tows during most of the summer.

The study area in the lower Mystic Estuary has been described elsewhere (Fell, 1974b); but because water temperature is of interest in considering the reproductive period of *Haliclona loosanoffi*, a summary of temperature readings taken over a five year period is presented in Figure 3. It should be noted that there is a rapid rise in water temperature during the late spring and that by early June the water temperature is generally above 20° C.

Pattern of larval settlement

The pattern of larval settlement on *Mercenaria* shells suspended in the Mystic Estuary was very striking. Nearly all of the settlement by the larvae of *Haliclona loosanoffi* occurred on the under (inner) surface of the shells (Fig. 2A). Since *Mercenaria* shells have inner and outer surfaces of very different textures, the preferential settlement on the inner surface could be a surface phenomenon. However, two pieces of evidence suggest that other factors are also involved. First, the free-swimming larvae, observed in the laboratory, became negatively phototactic before settling. The newly released larvae exhibited no definite phototaxis, but within 24 hrs at about 25° C a negative phototaxis was apparent. Secondly, it was shown that the larvae exhibit a distinct preference for settling on the under surface of *Mytilus* shells in the laboratory under conditions in which a preference for surface texture could be ruled out (Table II).

In the laboratory most of the larvae settled and formed small sponges with a single oscular tube within 48 hrs at about 25° C. The larvae attached at or near the anterior pole with the brown pigment ring pointing out. Under conditions in which there were large numbers of larvae settling on a limited surface area, many examples of postlarval fusion (usually in twos and threes) were observed.

DISCUSSION

Gametogenesis and embryonic development in *Haliclona loosanoffi* are essentially identical to these processes in *Haliclona ecbasis* and *Haliclona permollis* (Fell, 1969 and 1974a). Hartman (1958) described the eggs of *Haliclona loosanoffi* as ranging in size from 25 × 25 μ to 43 × 35 μ and the embryos as ranging in size from 150 × 130 μ to 215 × 185 μ. Evidently what he designated as eggs were small oocytes which had not yet begun the process of nurse cell engulfment and no distinction was made between the larger oocytes and the early-stage embryos. Growing oocytes are never very abundant and they may have been missed by him. However, the larger oocytes and early embryos contain large numbers of engulfed nurse cells and appear very similar at low magnification. This similarity is enhanced by the fact that distinct blastomeres usually can not be detected during the early cleavage stages.

In *Haliclona loosanoffi* the sexes are separate, a condition found in many other sponges (Fell, 1974a; Sarà, 1974); and the sex ratio is roughly 1:1. However, abortive small oocytes are seen in many male specimens. A similar situation has been described for *Hymeniacidon sanguinea* by Sarà (1961); but in the population of this species, males were much more abundant than females.

Previous studies on the reproductive period of *Haliclona loosanoffi* and a number of studies on the reproduction of other sponges have been largely or completely confined to an analysis of larval settlement. However, the amount of larval settlement observed may be the result of at least four different phenomena: 1) production of larvae by sponges inhabiting the region of study, 2) transport of larvae into and out of the study area by currents, 3) larval mortality, and 4) competition by other kinds of larvae for settling space. Therefore larval settlement curves do not necessarily give a true picture of reproductive activity and it is desirable to confirm the results of larval settlement studies by another method(s) of analysis. In the present investigation, an analysis of larval settlement was combined with a study of specimens collected at regular intervals from the same region. The results of these studies were in good agreement. Since larval settlement was found to increase during the period when the percentage of specimens containing large numbers of reproductive elements was falling (Fig. 2A), there is reason to believe that the larval settlement curve may accurately reflect reproductive activity in this case. The greatest larval settlement occurred in July.

Hartman (1958) studied larval settlement of *Haliclona loosanoffi* in Milford Harbor, Connecticut for three consecutive years and came to the conclusion that water temperature was an important factor influencing the onset of reproduction. At this location larval settlement occurred from August through October. Eggs and embryos were found in specimens collected in late August and early September and eggs alone were found in specimens taken in late September and early October. Wells, *et al* (1964) observed that at Hatteras Harbor, North Carolina larvae of this species settled during June and July and again in October and November. At both localities the first larval settlement occurred about two to four weeks after the water temperature rose above 20° C.

Although the reproductive period of *Haliclona loosanoffi* at Mystic, Connecticut may slightly overlap the reproductive period at Milford, it corresponds more closely to the major reproductive period at Hatteras Harbor (Fig. 3). The water temperature in the Mystic Estuary and Hatteras Harbor are very similar during the late spring, and this fact may account to a large extent for the similarities in the reproductive periods of *Haliclona loosanoffi* at these locations. The shallowness of the Mystic Estuary is probably the leading factor responsible for the early rise in water temperature compared to that in Milford Harbor (Pearcy, 1962). Since Mystic is only about 70 miles east of Milford on Long Island Sound, it seems highly probable that the differences in the reproductive period at these places is due to the observed differences in water temperature.

In the Mystic Estuary specimens of *Haliclona loosanoffi* with oocytes and embryos may be found in late May or early June, about two weeks earlier than those containing flagellated larvae. In addition, experiments, involving the placement of refrigerator-stored gemmules of *Haliclona loosanoffi* back into the Mystic Estuary, have shown that within about three weeks after the development of small sponges (at *ca.* 25° C) some of them may contain flagellated larvae (Fell, unpublished observations). These facts suggest that reproduction is probably initiated sometime between two and four weeks before the appearance of the first larvae and support the suggestion of Hartman (1958) that 20° C is close to the critical temperature at which the reproductive process begins (Fig. 3).

The factors responsible for the termination of reproductive activity in populations of *Haliclona loosanoffi* are probably several and depend upon local conditions. At Hatteras Harbor the first period of reproduction ends in July when the water temperature approaches 30° C (Wells, *et al*, 1964), and at Milford reproductive activity ceases in the fall when the water temperature falls below about 16° C (Hartman, 1958). In both cases the sponges are dying. The second reproductive period at Hatteras Harbor ends when the water temperature declines to below 16° C, but at this location many of the specimens of *Haliclona loosanoffi* survive the winter (Wells *et al*, 1964). In the Mystic Estuary actual reproduction ceases in late July or early August when the water temperature is about 22° C. At this time the sponges are actively engaged in gemmule production which normally continues for two more months (Fell, 1974b and 1976). There may be an antagonism between sexual reproduction and gemmule formation, perhaps in the form of competition for nutritive reserves.

A relationship between water temperature and the timing of sexual reproduction has also been demonstrated in *Hippiospongia lachne* (Storr, 1964). Reproduction of this species was studied at Cedar Keys, Florida, the Bahamas and British Honduras; and it was found that the peak of reproductive activity was reached earlier in the year in progressively more southern regions. Reproduction did not occur when the water temperature was below 23° C, and reproductive activity declined when it rose above 29° C.

It was shown here that the larvae of *Haliclona loosanoffi* tend to settle on under surfaces. This is consistent with the observed distribution of adults in the field. Hartman (1958) found that this sponge frequently encrusts the under surface of intertidal rocks, and Wells, *et al* (1964) observed that it typically occurs in obscure places where it is protected from the sun. *Haliclona loosanoffi* is sometimes found in large numbers encrusting the shells of mussels (*Mytilus edulis*) attached to the bottom of floating docks. Although the larvae become negatively phototactic before they settle, other aspects of their behavior may also be important to achieving the observed pattern of settlement. For example, in the strings of mollusc shells used in this study as spat collectors, the upper surfaces of most of the shells were shaded by the shells higher on the strings. The larvae may become negatively geotactic upon swimming into a shaded region and therefore settle almost exclusively on the under surfaces (see Crisp, 1974). There are also other possible explanations, and more extensive studies on the behavior of the larvae of *Haliclona loosanoffi* under a variety of conditions are needed.

In places where eel grass is present, *Haliclona loosanoffi* may be among those forms which settle on it in large numbers (also see Marsh, 1970). Even though the larvae may settle primarily on what are the under surfaces of the blades at the time of settlement, this orientation is obviously transient; and the postlarvae are found on upper, lower and vertical surfaces.

Appreciation is expressed to Sibyl Hausman and Jeanie Kitchen for expert assistance with the histological work and to Dr. Frances Roach and Ruth Fell for invaluable help with the field studies.

Summary

1. A general description of the reproductive elements of *Haliclona loosanoffi* is given. They were found to be very similar to the corresponding elements of other North American haliclonids.

2. *Haliclona loosanoffi* apparently is gonochoric, and the sex ratio is about 1 : 1. Abortive small oocytes are found in many male specimens during the latter part of the reproductive period.

3. The reproductive period of *Haliclona loosanoffi* in the Mystic Estuary, Connecticut is from late May or early June to late July or early August; but specimens with only small oocytes or spermatic cysts may be found until late September.

4. The reproductive period at Mystic, Connecticut is about two months earlier than at Milford, Connecticut and occurs at about the same time of year as the major reproductive period at Hatteras Harbor, North Carolina. Water temperature appears to be a major factor determining the reproductive period.

5. Very small specimens of *Haliclona loosanoffi*, as well as large ones, have high densities of reproductive elements during the early part of the reproductive period. Therefore it appears that size is not a major factor affecting reproductive activity in this species.

6. The larvae of *Haliclona loosanoffi* become negatively phototactic before they settle and exhibit a definite perference for settling on the under surface of submerged substrates. This pattern of settlement conforms to the distribution of adult specimens.

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CHLORIDE AND OSMOTIC BALANCE IN THE EURYHALINE SIPUNCULID *PHASCOLOSOMA ARCUATUM* FROM A MALAYSIAN MANGROVE SWAMP

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Studies on the ability of members of the exclusively marine phylum Sipuncula to regulate their ion and water content have been admirably reviewed by Oglesby (1968, 1969). Although body volume may be controlled under conditions of low stress, virtually all studies point to the absence of osmoregulatory ability among sipunculids. The only possible exception is the species Oglesby (1968, 1969) calls *Phascolosoma lurco.* The high mangrove swamp habitat of this species, subject to extremely variable salinities (an atypical situation for a sipunculid), suggests that it might be able to regulate ions and/or volume, and Gross (1954), analyzing data from a study on this worm by Harms and Dragendorff (1933) (who referred to the species as *Physcosoma lurco*), concluded that this was indeed the case. However, the evidence is open to other interpretations, and Oglesby (1969, page 233) stated, "Detailed investigation of this interesting worm would be rewarding."

Phascolosoma arcuatum (Gray, 1828), as it is now known [for recent discussions of this species' taxonomy, see Rice and Stephen (1970) and Stephen and Edmonds (1972)], occurs from India to Queensland, Australia, presumably always in mud, in or near a mangrove swamp. Harms and Dragendorff (1933) repeatedly emphasized that the worms they studied in Ambon and Sumatra, Indonesia, were found only at the highest levels of the mangrove swamp, in mud covered by sea water only at extreme high tides. We collected specimens for study from areas subject to daily tidal inundation, as well as from high sites. In a mangrove swamp 25 miles south of our collecting site, Sasekumar (1974) found *P. arcuatum* at levels tidally wetted from 26 to 365 days a year, although the species was most abundant ($39/m^2$) in an area wetted 210 days per year (approximately mean high water spring). S. J. Edmonds (University of Adelaide, personal communication), who confirmed the identity of our worms, stated that in Queensland they live near mid-high water level.

We present here data from baseline measurements on the osmotic and ionic content of the coelomic fluid of freshly collected worms; from experiments on the effects of the type of media on the osmotic and ionic content of the worms' coelomic fluid; and from an experiment in which worms were subjected to a variety of salinities for up to 64 hours, designed to study short-term responses to sudden declines in environmental salinity, such as might occur during a tropical rainstorm.

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MATERIALS AND METHODS

Specimens of *Phascolosoma arcuatum* were collected from the vicinity of Kuala Selangor, about 50 miles northwest of Kuala Lumpur, Malaysia. Subjects for experiments were all taken from the same area of mangrove swamp at the mouth of the Selangor River, where it was not necessary to dig deeper than 20–30 cm to find abundant animals. On one occasion coelomic fluid was drawn from the worms at the collection site and returned to the laboratory in individual tubes on ice. Thereafter intact worms were taken to the laboratory in a bucket of mud, no more than two hours elapsing between collection and arrival at the laboratory. Sampling was done, or experiments were begun, no more than 18 hours after collection, unless otherwise stated.

Mud was rinsed from the worms with tap water, the animals were blotted dry and weighed on a Mettler top loading balance (P160N) to the nearest 0.1 g. Worms less than one gram in weight contained insufficient coelomic fluid for our purposes; most specimens weighed between one and four grams, but some were as heavy as nine grams. Coelomic fluid was drawn into a syringe through a 25 gauge needle, the needle removed, and the contents dispensed into a plastic centrifuge tube. Samples were centrifuged for five minutes in a refrigerated Sorval SS-1 centrifuge at 3100 *g*. The plasma was decanted and frozen in small plastic vials at $-12°$ C until analyzed. In a number of samples some of the fresh fluid was drawn into a capillary tube and centrifuged at room temperature for five minutes in a hematocrit centrifuge. The length of the constituents in the tube was measured under a dissecting microscope and the percentage of formed elements calculated.

Chloride ion content, reported as milliequivalents per liter (meq), was determined by titration with 0.1 N silver nitrate (British Drug House Concentrated Volumetric Solution), using a potassium chromate indicator. Usually three but as many as five determinations were made on each sample to obtain a mean value. Osmotic concentration, reported as milliosmoles per liter (mosmol), was determined from measurements made on a Hewlett-Packard 302B Vapor Pressure Osmometer (VPO). A mean value was determined from a minimum of three but occasionally from four or five readings on a single sample. An effort was made to perform both chloride ion and osmotic concentration determinations on each sample. Conversion from titration or VPO values to chloride ion or osmotic concentration was by reference to a least squares linear regression line calculated from a series of sodium chloride solutions of known activity.

For the 64 hour experiment, about 160 freshly collected, rinsed animals were weighed and fluid was drawn from ten worms of various sizes. The others were put into individual plastic cups containing 100 ml 100% standard sea water [standard (artificial) sea water: NaCl 24.72 g/l; KCl 0.67 g/l; $CaCl_2 \cdot 2H_2O$ 1.36 g/l; $MgCl_2 \cdot 6H_2O$ 4.66 g/l; $MgSO_4 \cdot 7H_2O$ 6.29 g/l; $NaHCO_3$ 0.180 g/l (Cavanaugh, 1956)]. After 24 hours of equilibration they were reweighed, coelomic fluid was drawn from five specimens of various sizes, and the rest, still in individual cups, were divided into 35 groups of four worms each. An effort was made to have a distribution of worms of similar weights in each group. Seven groups of worms were covered with 100 ml of water of each of five salinities (100, 75, 67, 50, and 40 per cent of standard sea water). Coelomic fluid was drawn from one group in each salinity 1, 2, 4, 8, 16, 32, and 64 hours later. All remaining worms were weighed at each sampling time, as well as at 24 and 48 hours when their water was changed. The cups were covered, more to prevent escape than evaporative loss, and the experiment was done in an air-conditioned laboratory ranging in temperature from 25.5 to 27.5° C.

All values are reported as a mean ± one standard deviation, and statistical significance is determined by Student's *t*-test, except where otherwise noted. In the few instances that the standard deviation exceeded 10% of the mean, the divergent value was eliminated from consideration and the statistics were recalculated. In some cases we found that the worm showing such deviation from others subjected to identical conditions had appeared abnormal.

Results

Baseline data

The coelomic fluid of *Phascolosoma arcuatum* does not clot when drawn. Although usually pink in color, the fresh fluid of several animals bled in the field was yellow. The percentage of formed elements in the fluid of 35 worms collected on four different occasions was 4.3 ± 2.3%, with a range of 2.2 to 12.8%, and after centrifugation formed two light colored layers bracketing a thicker layer of red colored cells. Data from the occasional animals in which greenish eggs were present in the coelomic fluid are not included.

Chloride ion and osmotic content of coelomic fluid of six batches of sipunculids are related to that of their media in Table I. In the first group listed, worms were collected on the bank of a drainage ditch across the main road and several hundred meters from the Selangor River, but the water came from the ditch itself. In all other instances, the animals were from typical mangrove swamp habitats and seep water from the same microhabitat was collected for comparison. In the fourth batch listed, coelomic fluid was drawn 18, 24, and 48 hours after collection, but since readings on the three were stastically equal, the results from all samples were combined.

Table I

Chloride and osmotic content of coelomic fluid from freshly collected sipunculids and water from their immediate environment. Each line represents a single collection.

mosmol		Number of worms	CF/M	Significance of difference $P \leq$	meq Cl^-		Number of worms	CF/M	Significance of difference $P \leq$
Medium (M)	Coelomic fluid (CF)				Medium (M)	Coelomic fluid (CF)			
*342.7	632.5 ± 49.2	10	1.85	0.01	*138.2	240.2 ± 14.5	10	1.74	0.01
378.5	625.5 ± 50.5	8	1.65	0.01	192.3	335.2 ± 27.1	9	1.74	0.01
559.7	731.4 ± 31.8	7	1.31	0.01	253.4	349.4 ± 6.9	5	1.38	0.01
675.9	749.7 ± 65.3	15	1.11	n.s.	283.3	269.4 ± 24.0	18	0.95	n.s.
830.2	759.3 ± 38.8	17	0.91	n.s.	359.0	360.6 ± 14.9	21	1.00	n.s.
738.9	804.7 ± 35.8	5	1.09	n.s.	422.4	395.9 ± 39.3	6	0.94	n.s.

* Not seep water; see text.

TABLE II

Chloride and osmotic content of coelomic fluid of worms and their medium after being kept in either mangrove swamp water or mud, or in artificial sea water. Variances were compared by Bartlett's test for homogeneity of variances, the χ^2 values of which are indicated between the two values compared.

		mosmol		Number of worms	CF/M	Significance of difference $P \leq$	meq Cl^-		Number of worms	CF/M	Significance of difference $P \leq$
		Medium (M)	Coelomic fluid (CF)				Medium (M)	Coelomic fluid (CF)			
24 hr	mud	718.6	415.1 ± 21.7	3	0.58	0.01	215.2	198.5 ± 10.6	8	0.92	n.s.
		χ^2 = 1.69 (ns)					χ^2 = 1.26 (ns)				
	water	793.2	765.1 ± 46.1	6	0.96	n.s.	345.4	337.3 ± 14.7	6	0.98	n.s.
48 hr	mud	731.3	701.9 ± 6.0	4	0.96	0.05	356.9	330.2 ± 8.3	5	0.93	0.05
		χ^2 = 10.37 ($P < 0.01$)					χ^2 = 0.08 (n.s.)				
	water	563.6	546.6 ± 37.0	4	0.97	n.s.	245.9	228.6 ± 9.1	6	0.93	n.s.
24 hr	artificial sw	897.5	885.7 ± 36.4	5	0.99	n.s.	461.5	476.1 ± 10.4	4	1.03	n.s.
48 hr		1016.3	932.2 ± 54.7	5	0.92	n.s.	484.0	448.6 ± 38.4	4	0.93	n.s.
		1075.1	946.8 ± 14.0	8	0.83	0.01	428.5	457.4 ± 8.3	7	1.07	0.02

In another two cases, worms but not water were collected. Chloride ion content of these was 411.1 ± 31.5 meq (nine worms) and 493.5 ± 38.9 meq (20 worms). VPO measurements were 831.7 ± 51.1 mosmol (eight worms) and 990.4 ± 68.9 mosmol (18 worms), respectively.

Mud versus water medium

Experiments were done on two occasions to determine whether any osmoregulatory ability that might exist would be affected by keeping worms in water rather than in mud. Sipunculids, mud, and seep water were collected at the same time and place. In the laboratory the worms were rinsed and randomly placed for 24 hours in one case or 48 hours in the other, either in mangrove swamp water or in mud saturated with the same water. Among the specimens collected for the 48 hour experiment, some were bled immediately upon return to the laboratory. The upper part of Table II summarizes the findings of these experiments. Results of three other experiments, in which worms were kept in 100% standard (artificial) sea water for 24 or 48 hours, are summarized in the lower part of Table II.

In addition to the application of Bartlett's test for homogeneity of variances (Sokal and Rohlf, 1969) between coelomic fluid of experimental subjects as indicated in Table II, this test was used to compare variances between these fluids and those of fresh worms. The variance of the chloride values of neither the mud nor the water group of 48 hour worms differs significantly from that of fresh sipunculids ($\chi^2 = 0.27$ and 0.73, respectively), but variance in the VPO values of those kept in mud is significantly less ($P < 0.01$) than that of fresh specimens ($\chi^2 = 12.57$). Variance in coelomic fluid VPO values of fresh worms does not differ significantly from that of worms kept in water 48 hours (next to bottom line, Table II; $\chi^2 = 0.00$ and 1.40, respectively). In the 24 hour group, variance in VPO measurements between fresh and 24 hour coelomic fluid does not differ significantly ($\chi^2 = 1.26$), but the variance of the chloride ion values is significantly greater (at the 0.02 level; $\chi^2 = 5.79$) in coelomic fluid from fresh worms.

Salinity/time experiment

During the 24 hour equilibration period prior to the experiment, during which the worms voided most of their gut contents, 132 worms lost 22.3 ± 5.4% of their body weight, with losses ranging from 8.0 to 37.0%. Those weighing less than two grams after the 24 hours lost 23.6 ± 5.4% (N = 73), those in the range 2.0–4.4 g lost 21.5 ± 5.0% (N = 50), and those 4.5 g and more lost 17.5 ± 3.8% (N = 11) of their original body weight, the differences being significant.

Figures 1–3 and Table III present the results of this 64 hour experiment. To determine time to equilibrium, values from successive times were compared and in those cases where there was no difference at the 0.05 level of significance, alternate times were compared at the 0.02 level of significance. Worms were considered to have reached equilibrium when no significant differences were detected according to these criteria. For chloride ion and osmotic concentration readings, the mean value of all worms having attained equilibrium (*i.e.,* all worms at equilibrium time and all later times) was then compared with the value of the medium. The only exception to this was in the chloride ion concentration of worms kept in

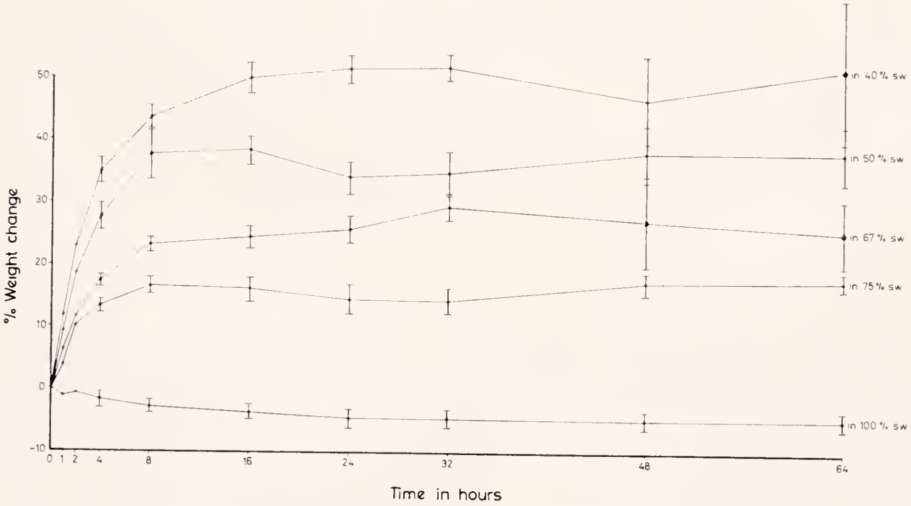

FIGURE 1. Change in weight of specimens of *Phascolosoma arcuatum* maintained in the laboratory for up to 64 hours in water of different salinities. One hour values are based on 22 worms in 100% sea water, 26 worms in 75% sw, 27 worms in 50% sw, and 28 worms in 40% sw. Numbers decline by three or four at each sampling time, and circled points represent a value obtained from only two worms. Standard error bars are omitted below four hours but are of a similar magnitude to the later ones.

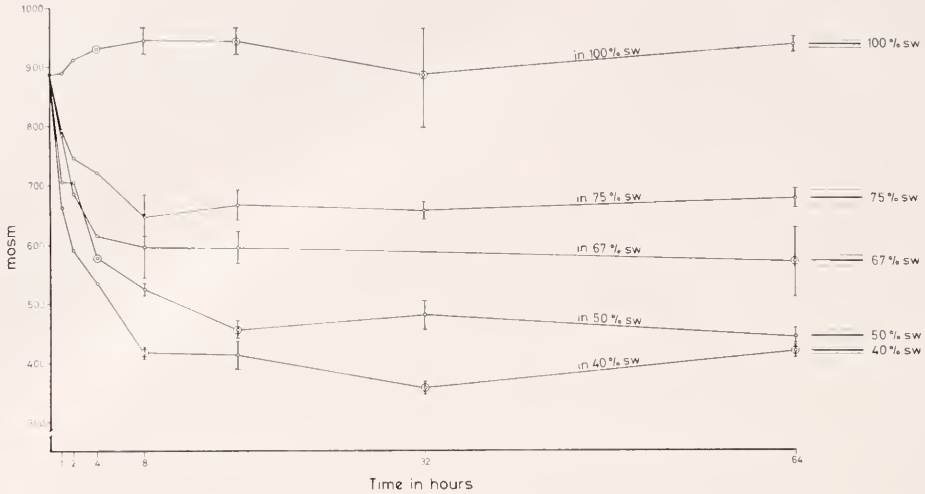

FIGURE 2. Changes in osmotic content of *Phascolosoma arcuatum* coelomic fluid with time. in water of different salinities. Circled points are means of measurements on two worms; other points are means of measurements on three or four worms. Standard deviation bars are omitted below eight hours but are of a similar magnitude to the later ones.

100% sea water. Values did not change significantly between successive times but the readings at two and eight hours differed at the 0.01 level of significance. Nonetheless a mean equilibrium value was calculated from all the readings. Similarly, the mean 100% osmotic concentration value was calculated from all readings (1–64 hours) since there was no significant change (neither between successive nor alternate times) through the course of the experiment. The osmotic concentration of worms kept in 40% sea water never attained a stable equilibrium, but all others did. Although a few of the experimental subjects escaped or died during the course of the experiment, the losses were not correlated with salinity.

DISCUSSION

The yellow color of the coelomic fluid in a few of the animals bled in the field was due to the deoxygenated state of the hemerythrin. The pink fluid of all those bled in the laboratory reflected the oxygenated condition of the pigment, showing that the time (in some cases only a few hours) between removal from the mud and bleeding was sufficient for oxygenation to occur. After centrifugation, the fluid was usually clear and colorless, although in a few cases it was cloudy and yellowish, but there was no correlation between its appearance and its osmotic character. Nor was there a correlation between the formed element content of a worm's coelomic fluid and its osmotic character.

Individual baseline measurements ranged from 189 meq Cl^- and 396 mosmol to 469 meq Cl^- and 963 mosmol, and up to 571 meq Cl^- and 1135 mosmol in animals along with which no seep water was collected for comparison. The proportion of the total osmotic concentration accounted for by chloride is quite variable,

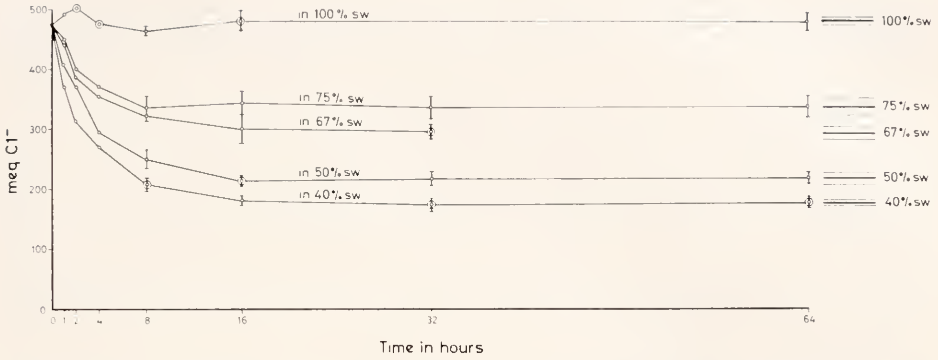

FIGURE 3. Changes in chloride ion content of *Phascolosoma arcuatum* coelomic fluid with time, in water of different salinities. Circled points are means of measurements on two worms; other points are means of measurements on three or four worms. Standard deviation bars are omitted below eight hours but are of a similar magnitude to the later ones.

Harms and Dragendorff's (1933) baseline values ranged from 470 to 1040 mM NaCl, although it was not always clear whether the value was obtained from the coelomic fluid of a single worm or from a pooled sample. Although such a range is apparently not found in natural populations of other species of sipunculids so far studied, Oglesby (1968) reported a range of approximately 100 to 600 mM NaCl for groups of the stenohaline sipunculid *Themiste dyscritum* after laboratory adaptation to various salinities.

The osmotic and chloride ion content of collected seep water varied from about 42 to 92% of standard sea water (excluding water values in the first line of Table I). *Phascolosoma arcuatum* is hyperosmotic and hyperionic in waters as concentrated as 253 meq Cl^- (55% standard sea water), and 560 mosmol (62% standard sea water). In more concentrated media, there is no significant difference between chloride or osmotic content of coelomic fluid and that of the medium, although data from two of the three groups indicate that the worms may be hyperosmotic even at higher salinities. Coelomic fluid of most of the worms studied by Harms and Dragendorff (1953) was hyperosomotic to the medium. Curiously,

TABLE III

Time to equilibrium after 24 hours pretreatment in 100% sea water, and ratio of coelomic fluid to medium osmotic and chloride ion concentration at equilibrium.

Percentage of sea water	Weight	Osmotic concentration		Chloride ion concentration	
	Hours	Hours	CF/M	Hours	CF/M
100%	—	—	1.02	—	1.04
75%	8	8	0.98	8	0.97
67%	8	2	1.00	8	1.01
50%	8	16	0.99	8	1.00
40%	16	—	—	16	1.02

it appears that conforming worms can have a lower chloride content than those in which the Cl^- level is significantly greater than that of the medium (*e.g.*, compare the fourth batch, Table I, with the preceding two). Further studies are necessary to clarify these points.

In the time/salinity experiment, on the other hand, worms at all experimental salinities reached equilibrium well within 64 hours, at which levels osmotic concentration (except for the 40% group) and chloride ion content did not differ significantly from those of the media. In fact, although our 100% sea water was more concentrated than any of the waters collected *in situ,* 24 hours in it was sufficient for the worms to conform to it, as evidenced by the lack of significant change in Cl^- and VPO values in the 100% groups of worms during the succeeding 64 hours of the experiment.

The discrepancy between isosmosity of coelomic fluid of nearly all groups of experimental worms and hyperosmosity of coelomic fluid of at least some groups of freshly collected animals may be in part a reflection of methodological problems. Seep water might not accurately reflect the immediate osmotic environment of the worms, a possibility also discussed by Harms and Dragendoff (1933). They repeatedly emphasized that *P. arcuatum* lives in air-filled tubes so that the worms are not actually in contact with the ground water, adding that the worms avoid water and cannot even be kept in it because they are adapted to aerial respiration. The deoxygenated hemerythrin that occurred in some of our worms bled in the field suggests that their tubes were probably water-filled. Possibly the coelomic fluid hyperosmosity was due to desiccation, as suggested by Oglesby (1969, page 233), since all our specimens were collected at low tide, but if this were the explanation, the phenomenon probably would be evident at all salinities. It is also possible that at low tide, water from the near-by Selangor River percolated through the mud, lowering the salinity of the seep water. Thus worms collected at this time may not have reached equilibrium.

Since it is easier to define salinity for experimental purposes in an aqueous medium than in mud, and since we could be certain that in water the animals would be in contact with the medium we had defined, we preferred to use a water medium, providing it did not interfere with the worms' normal physiological processes. In the mud versus water medium experiments, only one of five groups of worms kept in water differed in chloride and osmotic content from their medium, whereas comparable values for coelomic fluid of the two groups kept in mud were both lower than water derived from the mud (although the difference was not significant for Cl^- of the 24 hour group). If the sipunculids were "drowning" in water, their physiological integrity might be expected to deteriorate, but there was no evidence of this. In only one instance was a significant difference found between the variances of coelomic fluid measurements of those kept in water and of those kept in mud, but comparison with fresh coelomic fluid of worms collected at the same time showed no difference in variances of osmotic content with that of worms kept in water for 48 hours. Thus the variability of the coelomic fluid of those animals kept in water was not greater than usual, but that of those kept in mud was less than usual. The only other instance of significantly different variances was that Cl^- of fresh worms was greater than that of worms kept in artificial sea water for 24 hours, but variances in osmotic concentration of the same worms were statistically equal.

Thus it appears that at least for the length of time involved in these studies, immersion in water does not affect the osmotic physiology of *Phascolosoma arcuatum*. Indeed, as it turned out, nearly all worms in the 64 hour experiment showed normal muscle tone and activity even in 40% sea water and for up to 64 hours.

Results of the 64 hour experiment clearly indicate that *P. arcuatum* can be an osmo- and chloride ion-conformer, but it is also apparent from Table II that a mud medium may permit the worms to differ from their environment, assuming that water obtained by centrifuging this mud is an accurate reflection of that environment. For example, seep water of the third group in Table I is of nearly the same salinity as that of water in the 48 hour mud/water experiment (Table II) but coelomic fluid of the former worms is significantly higher in both parameters than the medium whereas in the latter they are statistically equal. Tubes built by worms in mud might isolate the animals from the seep water. *P. arcuatum* is a deposit feeder and freshly collected worms always had guts filled with mud. Our data suggest that the presence of substrate within and without the animal may modify the organism's response to osmotic stress. A mud-filled gut might serve as a buffer against changes in the external environment, perhaps being especially important during short-term changes in environmental salinity, such as during rainstorms at low tide or during a period of unusually high tides. An assessment of the relative roles of body wall and gut wall in water and ion transport is clearly needed.

The fact that time to ionic equilibrium in the 64 hour experiment was roughly the same as the time to weight equilibrium suggests that the movement of water was responsible for the adjustment. In contrast with the situation in *Physcosoma japonicum* (Koller, 1939) where worms transferred to lower salinities return to their original weight within 30 hours after an initial 10 hour period of weight gain, in *P. arcuatum* there was no return to some "baseline" level. Our data conform to Gross' (1954) generalization that time to osmotic equilibrium in sipunculids is directly related to the extent of the osmotic stress, and suggest that large worms take longer to reach equilibrium as also noted by Gross, although numbers of animals in each time/salinity group were too small to analyze statistically for differences that might be due to their weight. Our finding that during the 24 hour equilibration period, larger worms lost proportionately less weight, suggests that there are probably differences in osmotic behavior in worms of various sizes. (This initial weight loss was due in part to voiding of feces. However, the 100% sea water in which the worms were acclimated was more concentrated than that from which they had been collected and since, as it turned out, the worms were osmo-conforming, presumably part of the weight loss was due to water loss.) Further evidence for this is that, of the ten values that were discarded in the 64 hour experiment because they made the s.d. greater than 10% of the mean, seven were from the largest worm of a group of four, and all were values much lower than the others of the same group.

As mentioned in the introduction, *Phascolosoma arcuatum* occupies a much wider intertidal range in Malaysian mangrove swamps than is reported by Harms and Dragendorff (1933) for the species in Indonesia. The fact that they used individuals collected so high in the swamp may account for some differences between their observations and ours. In addition, their sample sizes were very small, limiting the usefulness of the study. Results of our research lead us to conclude that

Phascolosoma arcuatum, like all other sipunculids studied in this way, is physiologically an osmoconformer, and thus, in water, this species is really no different from *Themiste dyscritum,* which is also tolerant of and can conform to salinities ranging from about 40 to 100% (Hogue and Oglesby, 1972), although the former is naturally exposed to such a variable environment and the latter is not. However, under natural conditions, methods of behaviorally manipulating its environment may be a factor in allowing *P. arcuatum* to be hypersomotic to its environment, at least at low salinities, and this distinctive feature probably contributes to this species' ability to live in its atypical habitat.

We are grateful to Prof. A. J. Berry for introducing us to *Phascolosoma arcuatum,* to Dr. S. J. Edmonds for identifying the worms, to Mr. M. Arunasalam for digging them, and to Mr. Amir bin Salleh for drawing the graphs. Drs. A. Pritchard, Y. C. Siew, R. I. Smith, and G. C. Whittow gave helpful suggestions. This work was supported by a research grant from the University of Malaya.

SUMMARY

1. The euryhaline sipunculid *Phascolosoma arcuatum* (Gray, 1828) occurs from lowest to highest levels of mangrove swamps along the west coast of Peninsular Malaysia.

2. The chloride ion content of coelomic fluid of freshly collected *P. arcuatum* varied from 189 to 571 meq Cl^-, and the total osmotic content varied from 396 to 1135 mosmol. The lower concentrations were hyperionic and hyperosmotic to the surrounding water, but the higher concentrations of coelomic fluid were isoionic and isosmotic.

3. Animals kept for up to 64 hours in the laboratory in artificial sea water ranging from 40 to 100% standard sea water were uniformly isoionic and isosmotic at equilibrium. Weight stabilized at about the same time as did ionic and osmotic content of the coelomic fluid, without evidence of being regulated.

4. Under natural conditions, mud in the worms' guts and around them may act as a buffer, thereby allowing the animals to maintain an ionic and osmotic state differing from that of their environment.

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INTERACTIONS BETWEEN MOLTING AND REGENERATION IN THE LAND CRAB

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This laboratory has previously called attention to the efficacy of loss of a critical number of limbs, either pereiopods or chelipeds, in stimulating precocious molts in Crustacea (Skinner and Graham, 1970; 1972). The underlying mechanism for that stimulation is not yet known. The interval between limb loss and ecdysis is somewht longer than that undergone by animals triggered to molt by loss of eyestalks, which contain the neurosecretory X-organ sinus gland complex, the source of an as yet incompletely characterized molt inhibitory hormone (Bliss, 1956; Passano, 1960; Rao, 1965). In most species, animals that have undergone precocious molts initiated by eyestalk removal die at or immediately following ecydsis. By contrast, mortality following limb loss is very low (Skinner and Graham, 1972). In recent years, removing a critical number of limbs has become a common means of inducing precocious molts.

Although the minimal effective stimulus in the land crab, *Gecarcinus lateralis,* is five or more limbs, others have shown that loss of four limbs from the freshwater shrimp, *Palaemonetes kadiakensis,* at all stages of the molt cycle except the late premolt period decreases the duration of the intermolt period by as much as 40% (Stoffel and Hubschman, 1974). Similarly, the number of animals molting within a given time period is directly correlated with the number of walking legs removed from the edible crab, *Cancer pagurus* (Bennett, 1973) and from the fiddler crab, *Uca pugilator* (Fingerman and Fingerman, 1974). The latter authors also showed that in specimens of fiddler crab triggered to undergo precocious molts by loss of eyestalks, subsequent autotomy of walking legs had the converse effect: the duration of the premolt period was significantly *lengthened* in animals from which three or more limbs were autotomized as compared to animals missing eyestalks only or missing eyestalks and one or two limbs.

The present paper describes another set of interactions between regeneration and molting: loss of one or more partially regenerated limbs (primary regenerates) before a critical stage in the premolt period temporarily, for 10 to 14 days, decreases both growth and DNA synthesis of other primary regenerates remaining on the animal while the early stage of re-regeneration is initiated at the sites of the missing limbs. (Throughout the paper, we shall designate as primary regenerates those that form after autotomy of limbs and secondary regenerates those that form after autotomy of primary regenerates.) The duration of the premolt period is also lengthened for 10 to 14 days. Under these conditions, stimulation of growth (in the secondary regenerates) and inhibition of growth (in the remaining primary regenerates) are observed simultaneously in the same animal.

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The inhibition caused by the loss of regenerates is seen in animals stimulated to undergo precocious molts by either limb or eyestalk removal.

Several preliminary reports of these results have been published (Holland and Skinner, 1974a, b).

Materials and Methods

Animals

Specimens of *Gecarcinus lateralis* were obtained from the Bermuda Biological Station or from J. Van Montfrans or W. Miley, Boca Raton, Florida. Precocious molts were initiated by either removal of both eyestalks or induction of autotomy of eight walking legs (Skinner and Graham, 1970, 1972). Autotomy is the physiological self-amputation of injured limbs at a preformed breakage plane by the abrupt contraction of a set of autotomy muscles in the basi-ischial segment (Wood and Wood, 1932). Animals autotomize limbs when threatened or when the merus segment of the limb is injured. Regeneration of the autotomized limbs occurs early in the premolt period [stage D_0 (Skinner, 1962) and D_1, these data]. The data on the size of the regenerating limb buds are expressed as a regeneration index (R), which is the length of the regenerate (measured at 2.75 × magnification) divided by the maximal width of the carapace × 100 (Bliss, 1956). Because the length of regenerates of ($R \leqq 10$) is difficult to determine precisely, these smallest R values are approximate.

DNA synthesis

For *in vitro* studies, crabs were caused to autotomize regenerating limbs. The regenerates were rinsed several times in sterile 0.5 M NaCl and placed in chambers containing Eagle's Minimal Essential Medium with electrolytes adjusted to concentrations isotonic with *Gecarcinus hemolymph* (Skinner, Marsh and Cook, 1965) and 10% calf serum (Fig. 1). The composition of the medium is listed in Table I.

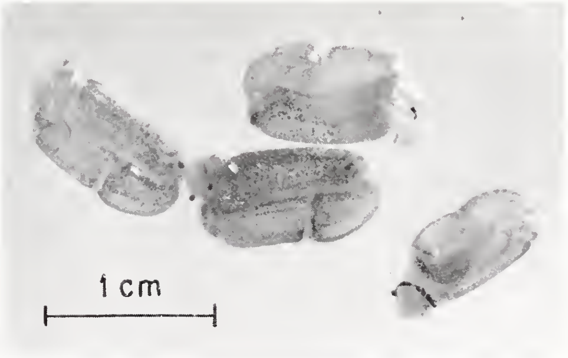

FIGURE 1. Four limb regenerates after autotomy, maintained *in vitro*.

TABLE I

Medium used for maintenance of limb regenerates in vitro.

Powdered MEM	9.51 g
*"100X" Amino acids	30 ml
100X Vitamin	30 ml
100X Glutamine 200 mM	20 ml
10% Na citrate	40 ml
100X Pyruvate (0.1 M)	10 ml
100X Antibiotic mix	10 ml
4 M NaCl	50 ml
0.7 M $CaCl_2$	10 ml
0.06 M $MgSO_4$	100 ml
Glass-distilled H_2O	540 ml
1 M NaOH to adjust pH to 7.3	8.6 ml
H_2O to	900 ml
Fetal calf serum or crab serum, added after filter sterilization to make 10%	100 ml

* Ten milliliters of the "100X" concentrates are normally used for 1000 ml of standard mammalian growth medium. As noted in the right-hand column, we have in some instances used more than this amount for crab tissues. MEM is Eagle's Minimal Essential Medium.

Crystals of phenylthiourea were added to inhibit tyrosinase activity (Harvey and Williams, 1961). Antibiotics were added to final concentrations of 100 units/ml penicillin, 0.25 μg/ml fungizone, and 100 μg/ml streptomycin. ^{3}H-thymidine (specific activity, 20 Ci/mmole) was used at 10–20 μCi/ml culture medium to label DNA. Chambers were rocked at 24 excursions/min at room temperature. The gas phase was air.

DNA was isolated from regenerates by methods described previously (Skinner, 1967) and displayed on two-step "relaxed" preparative neutral CsCl density gradients (Brunk and Leick, 1969). Specific activity of the DNA was determined by monitoring at 260 nm the absorbency of fractions dripped from a hole in the bottom of the gradient tube and by counting aliquots of selected fractions precipitated on Whatman No. 3 filters with 5% TCA (Bollum, 1959). To monitor the uptake of radioactive precursors into cell water, radioactivity in the ethanol-soluble fractions of the DNA preparations was determined by counting aliquots in dioxane.

The crustacean molt cycle

The crustacean molt cycle was divided into four major stages by Drach (1939). We present here a brief description of the molt cycle, of limb regeneration and of their temporal relationship in relatively large specimens of *Gecarcinus*. Stage A immediately follows ecdysis; it is very brief (1 to 2 days). In Stage B, 2 to 5 days, synthesis of endocuticle layers begins and continues into Stage C, during which the exoskeleton hardens further by calcification. The completion of the membranous layer, the innermost layer of the exoskeleton, concludes Stage C_3; Stage C_4, or intermolt period, is the longest part of the cycle, 10 to 11 months in duration, while the premolt period, Stage D, lasts 1 to 2 months. Regeneration of missing limbs occurs only during the premolt period in *Gecarcinus* (Bliss, 1956;

Hodge, 1958; Skinner, 1962) as in many other crustaceans (Emmel, 1910; Bliss, 1960; Adiyodi, 1972).

When a limb is autotomized, a scab is formed by clotted blood and the autotomy membrane that covers the breakage plane (Emmel, 1910; Hodge, 1958). Within two weeks the scab is erupted by a papilla which grows approximately 2 mm and then, in unstimulated (intermolt) animals, enters a state of quiescence, the basal growth plateau, that can last as long as ten months. In 1 mm limb regenerates, tissue differentiation is accompanied by segmentation (Hodge, 1958). Such miniature limbs with clearly defined segments are mobile and can be drawn close to the body. We find that 3-mm long regenerates can be autotomized if stimulated, indicating that functional connections have been re-established to the autotomy muscles located in the basi-ischium of the old limb. During the premolt period, the regenerate grows linearly until one to two weeks before ecdysis when it enters a second growth plateau. At this stage (D_1), the epidermis covering the branchiostegite regions of the carapace separates from the old exoskeleton. The cells enlarge and synthesize the new epi- and exocuticular layers comprising the premolt portion of the exoskeleton (Stage D_2) with which the animal emerges at ecdysis. Formation of exoskeleton on other parts of the body occurs at this time or before, as in the case of epipodites (Tchernigovtzeff, 1959). Since the duration of the premolt period varies from animal to animal, these cytological changes in the epidermis are used for more precise staging. A more complete summary of premolt events that subdivide Stage D into four discrete phases is included in Skinner (1962).

RESULTS

Growth patterns of regenerates of animals with or without eyestalks

The first experiment shows the effect of the presence or absence of eyestalks on the growth pattern of regenerating limbs. In the experiment depicted in Figure 2a, three animals from which eyestalks had been removed one or two days previously and three animals with eyestalks were caused to autotomize eight walking legs on day 0. Regenerating limbs were measured and their *R* values calculated as described. The curve to the left in Figure 2a shows the pattern of regeneration in animals missing eyestalks; that to the right, in animals with eyestalks.

In animals stimulated to molt by either eyestalk removal alone or by a combination of eyestalk removal plus limb autotomy, an autotomized limb is replaced by a papilla which grows linearly *without* entering the basal growth plateau characteristic of intermolt animals. The pattern of growth of regenerates of animals with eyestalks is similar, though not identical, to that of animals without (Fig. 2a). In both groups of animals blastema formation as well as the growth plateau immediately preceding ecdysis are of approximately 10 to 15 days' duration. The rate of growth of regenerates of animals without eyestalks is more rapid and the preecdysial growth plateau in such animals occurs when the regenerates are smaller ($R = 19$) than in animals induced to molt by limb autotomy ($R = 25$). Correlated with these two factors, the length of the premolt period of animals induced to molt by eyestalk removal is considerably shorter than that of animals induced to molt by limb autotomy (30 to 35 days as compared to 50 to 55 days; Fig. 2a).

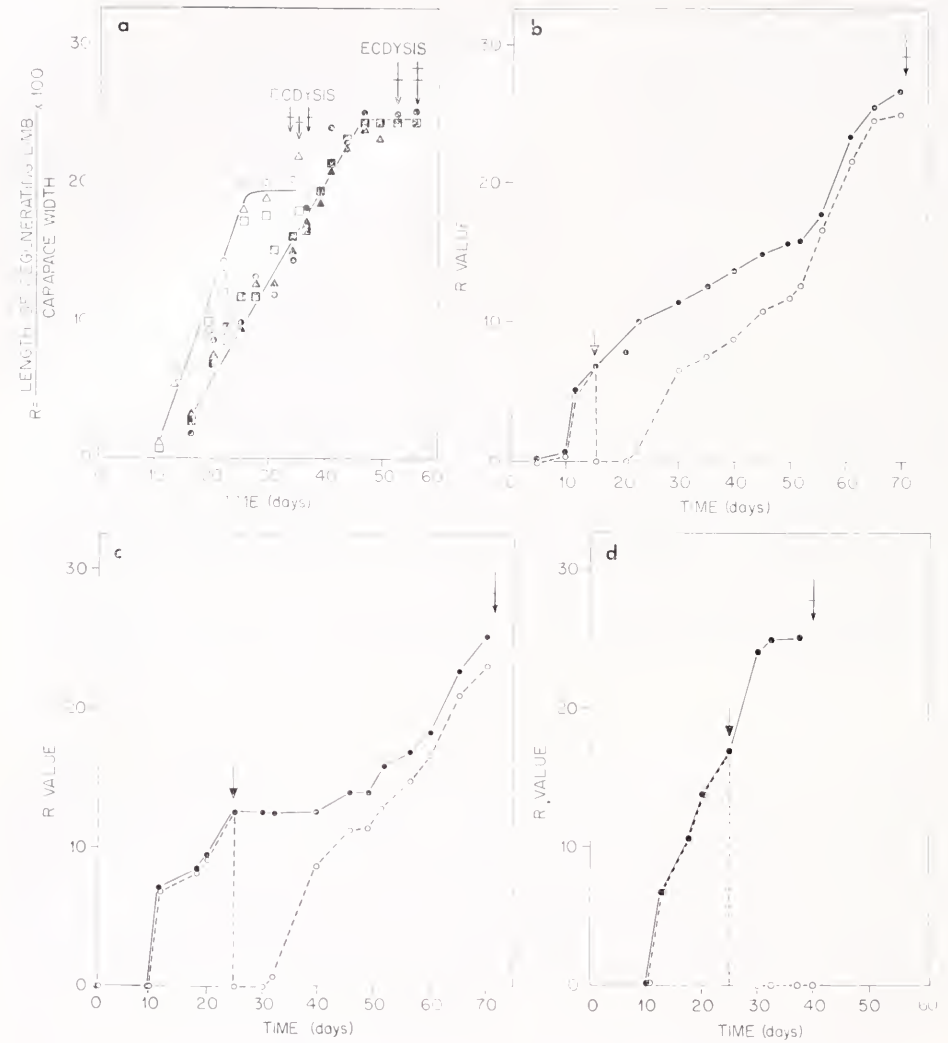

FIGURE 2. Pattern of growth of limb regenerates. Animals were caused to autotomize eight walking legs on day 0. Regenerating limbs were measured and their R values calculated as described in Materials and Methods. (a) R values of regenerates in three animals with (closed triangles, squares and circles) and three animals without (open triangles, squares and circles) eyestalks. Ecdysis of each animal is indicated by a crossed arrow. R values of (b) small, (c) medium, and (d) large regenerates are indicated by closed circles joined by solid lines. The time of autotomy of primary regenerates is indicated by the first arrows. The open circles joined by dotted lines indicate the R values of the secondary regenerates that form following autotomy of primary regenerates.

The growth patterns of primary and secondary regenerates

The effect of loss of partial primary regenerates on the growth of others remaining on the animal at various stages of regeneration has been determined. Animals were stimulated to undergo precocious molts by autotomy of eight walking legs (Time 0; Fig. 2b, c, d). Four partial regenerates were autotomized (arrow). The growth of one of the partial regenerates remaining *in situ* (primary regenerate, solid lines, Fig. 2b, c, d) was monitored at the same time as was the growth of one of the re-regenerating limbs (secondary regenerate; dashed lines, Fig. 2b, c, d).

The following observations were made. First, subsequent to autotomy of one or more small regenerates ($R = 7$ to 10), the growth rate of the remaining primary regenerates decreases markedly (Fig. 2b). Secondly, following autotomy of larger regenerates, growth of remaining primary regenerates ceases for 7 to 14 days (Fig. 2c). In both cases, a papilla grows from the base of each of the autotomy planes. When the growing secondary regenerates approach the same size as the primary regenerates, the primary regenerates resume growth at a rate similar to that of the secondary regenerates re-establishing the synchronized growth of all regenerates. Just prior to ecdysis the secondary regenerates are slightly smaller than the primary, as are the newly re-regenerated limbs after ecdysis. The merus segment of secondary regenerates is 10 to 15% shorter than that segment in primary regenerates and the weight of secondary regenerates is 10 to 20% lower (data not shown). Autotomy of a fully-formed nonregenerating pereiopod inhibits growth of primary regenerates as in the first and second cases. Third, autotomy of very large regenerates ($R \geqq 20$; stage late D_1 or later), has no effect on the remaining primary regenerates. The autotomized limbs are not regenerated (Fig. 2d) and the animal emerges from ecdysis without its full complement of limbs.

To determine if the inhibition of growth of partial regenerates in *Gecarcinus* was dependent on the autotomy of a critical number of regenerates, as is the initiation of a precocious molting period by limb loss, one primary regenerate was autotomized from three animals and two primary regenerates were autotomized from four other animals, all in stages D_0 to D_1. In every case, growth of the remaining primary regenerates was inhibited while secondary regenerates formed (data not shown). The autotomy of one primary regenerate is as effective as the loss of four or more regenerates in inhibiting growth of other primary regenerates.

Effect of secondary regeneration on the duration of the premolt period

It can be noted in Figure 2 that where growth of primary regenerates slowed (Fig. 2b) or stopped (Fig. 2c) the total premolt period was approximately 70 days; in the case where no inhibition of growth of the primary regenerates occurred (Fig. 2d), ecdysis took place on the forty-second day. A summary of data from a larger population of animals is given in Figure 3. In these experiments, as before, the molting period was initiated by autotomy of eight walking legs (Day 0) and at some later time four primary regenerates were autotomized. Growth of the primary regenerates was monitored and the time of ecdysis noted. The animals in Group 1 were those in which growth of primary regenerates was slowed while secondary regeneration occurred (see Fig. 2b); the animals in Group 2 were those in which growth of primary regenerates stopped while secondary regenerates formed (see Fig. 2c). The animals in Group 3 were those in which growth of primary regenerates was unaffected and there was no secondary regeneration (see Fig. 2d). In Group 3, ecdysis occurred at the same time as in controls (data not

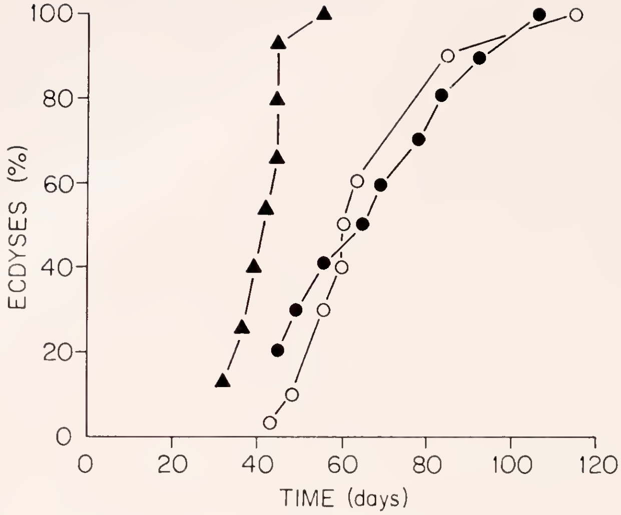

FIGURE 3. Duration of premolt periods of animals with or without re-regenerated limbs. In group 1, 4 of 8 partial regenerates were autotomized from ten animals early in the premolt period ($R = 7$–11). The time at which the animals underwent ecdysis, plotted as percent ecdysis, is indicated by open circles. Primary regenerates were autotomized from animals in Group 2 at $R = 13$–20 (closed circles). Primary regenerates were autotomized from animals in Group 3 in Stage D_2 or later ($R \geqq 20$; closed triangles). All twenty animals in Groups 1 and 2 formed secondary regenerates before ecdysis; those in the third group did not.

shown) from which primary regenerates had not been removed. Groups 1 and 2 in which the autotomy of regenerates was induced about one week earlier than Group 3 were delayed three weeks or longer in the time to ecdysis. It is concluded that the growth inhibition is correlated with a prolongation of the premolt period. This conclusion confirms and extends earlier observations (Skinner and Graham, 1972).

DNA synthesis during limb regeneration

In order to study the DNA synthetic activity of the limb regenerates under controlled conditions, autotomized regenerates were placed *in vitro*. In this way the chemical milieu as well as the specific radioactivity of the ^{3}H-thymidine precursors could be controlled and standardized. ^{3}H-thymidine levels within the tissues were constant, as noted below. The limb regenerates were incubated from 14–16 hr, during which time the rate of labeling remained constant (data not shown).

DNA was isolated and displayed on CsCl density gradients. The characteristic poly d(A-T) satellite and main component DNAs were seen (Skinner, 1967); the G+C-rich satellite was sometimes not detected because of the small amount of DNA centrifuged as well as its low thymidylate content. Some representatives of typical DNA preparations are shown in Figure 4. ^{3}H-Thymidine was incorporated into satellite (arrow) and main component DNAs at the same specific activities

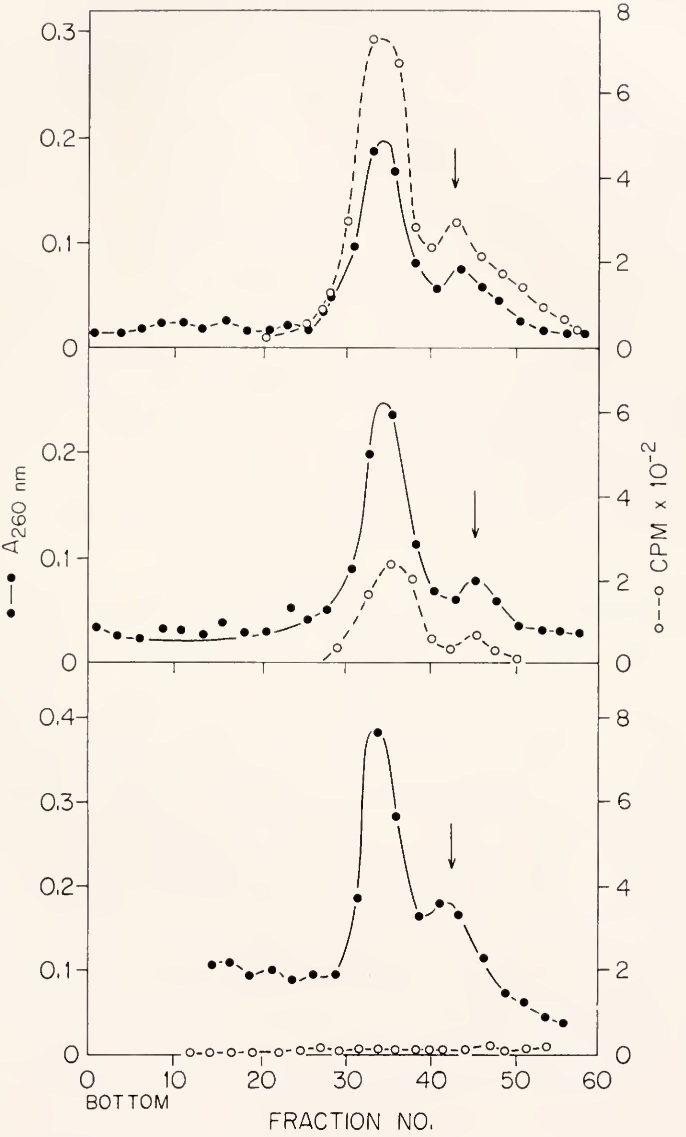

FIGURE 4. Preparative CsCl gradients of DNA from regenerates at different stages of the premolt period. Regenerates were incubated *in vitro* with ^{3}H-thymidine and the DNA was isolated and centrifuged in CsCl gradients. The poly d(A-T) satellite is indicated by the arrow. Fractions were collected, their UV absorbency at 260 nm was monitored, and the TCA-precipitable radioactivity of alternate fractions was determined: (A) early ($R=7$); (B) middle ($R=12$); and (C) late ($R=24$) stages of the premolt cycle. The closed circles and solid line indicate absorbency at 260 nm; open circles and dotted line indicate ^{3}H-thymidine.

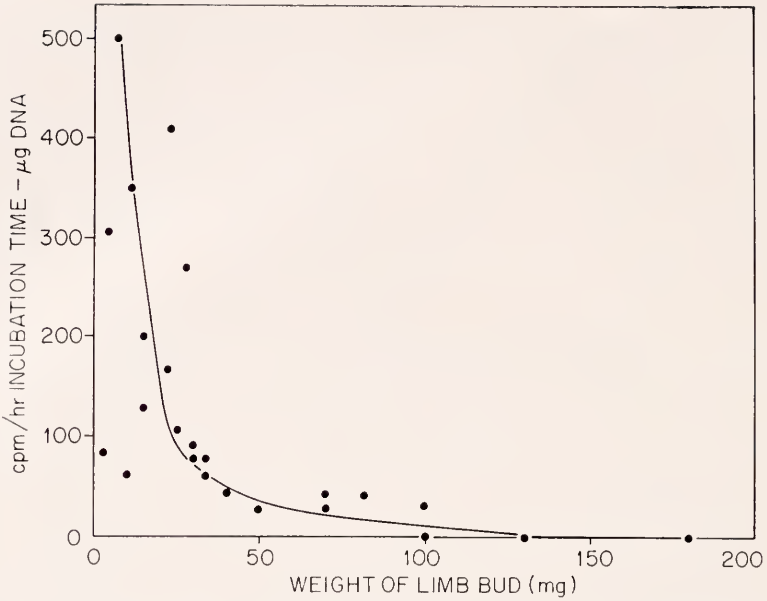

FIGURE 5. Incorporation of ^{3}H-thymidine into DNA during limb regeneration. The specific activity of main component DNA isolated from limb buds of different sizes and treated as described in Figure 3 is plotted versus weight of the regenerate.

when corrected for the different thymidylate contents of each. The DNA in the upper panel was isolated from a set of small, early, primary regenerates ($R = 7$), that in the middle panel from a set of larger regenerates ($R = 12$) taken later in the premolt period and that in the lower panel from large, late regenerates ($R = 24$). The amount of DNA centrifuged was approximately the same in each case. The incorporation of ^{3}H-thymidine is clearly greatest in the earlier regenerates and was indetectable in the large late regenerates. A summary of data from 24 experiments is given in Figure 5. As before, the greatest incorporation (counts/min/μg DNA) occurred in the smallest regenerates (Fig. 5). Incorporation of ^{3}H-thymidine fell to zero in fully formed regenerates (approximately 12 mm in length and 200 mg in a 60-gm animal).

Several weeks before ecdysis (late D_2), the regenerates become pigmented as the epicuticle is synthesized and secreted between the tissue and the sac covering the regenerate. To determine whether the epicuticle that forms on larger regenerates decreases their permeability to radioactive precursors, the radioactivity in cell water was determined by counting the ethanol-soluble fraction of DNA preparations from animals in the early, middle, and late stages of the premolt period. There were insignificant differences in uptake of precursors throughout the period of regeneration (Table II).

DNA synthesis in growing vs. inhibited regenerates

In parallel with the experiments showing that loss of one or more regenerating limbs markedly decreased the growth rate of other primary regenerates, we

TABLE II

Uptake and incorporation of ^{3}H-Thymidine in regenerates.

Stage in premolt period	*R* Value	DNA (cpm × 10^{-3} μg)	Ethanol-soluble radioactivity (cpm × 10^{-5}/g tissue)
Early*	8	3.23	2.80
Middle†	12	1.10	2.52
Late‡	22	0.0009	2.73

* Early = D_0; regeneration of limbs, formation of gastroliths.

† Middle = D_0 to D_1; as above until D_1, separation of epidermis from old exoskeleton (apolysis; Jenkin, 1970).

‡ Late = Animals in stage D_2 (exoskeleton synthesis).

measured the degree of inhibition in the rate of DNA synthesis. In these experiments, autotomized limb buds were used for the zero time controls to establish the level of incorporation of precursors into DNA. The removal of the control limb buds also served as the event leading to the inhibition of growth, and, as will be seen, inhibition of DNA synthesis in remaining regenerates. The remaining regenerates were autotomized 48 hr later and assayed for their rate of DNA synthesis.

In a series of control experiments, the rate of DNA synthesis of four regenerates was compared with the rate of synthesis of the four bilaterally symmetrical regenerates autotomized from the same animals at the same time. As shown in Table III, the rates did not differ by more than 20%.

Four small regenerates were autotomized and their relative rates of DNA synthesis determined *in vitro*. The main band DNA had a specific activity of 4.7 × 10^3 cpm/μg (Fig. 6a). The four regenerates remaining on the animal, autotomized 48 hr later, had a specific activity of 10 cpm/μg (Fig. 6b). A similar, almost complete cessation of DNA synthesis was seen in nine separate experiments (Table IV-A). In each case, insignificant differences were found in the radioactivity in the cell water, and in each case ecdysis was delayed while secondary regenerates were formed.

To determine whether the inhibition of DNA synthesis induced by loss of partial regenerates was stage-specific, similar experiments were performed with smaller ($R = 8$) and larger ($R = 24$) partial regenerates. A set of four smaller regenerates autotomized on day 0 had a synthetic rate approximately equal to that

TABLE III

Normal variability in DNA synthesis of limb regenerates.

R	SA (a)* (cpm × 10^{-3}/μg DNA)	SA (b)* (cpm × 10^{-3}/μg DNA)	SA (b)/SA (a)
18	2.35	2.18	0.93
14	13.86	12.05	0.87
13	5.58	5.31	0.95
10	6.65	5.59	0.84

* SA (a, b)—specific activity of two groups of regenerates removed on day 0.

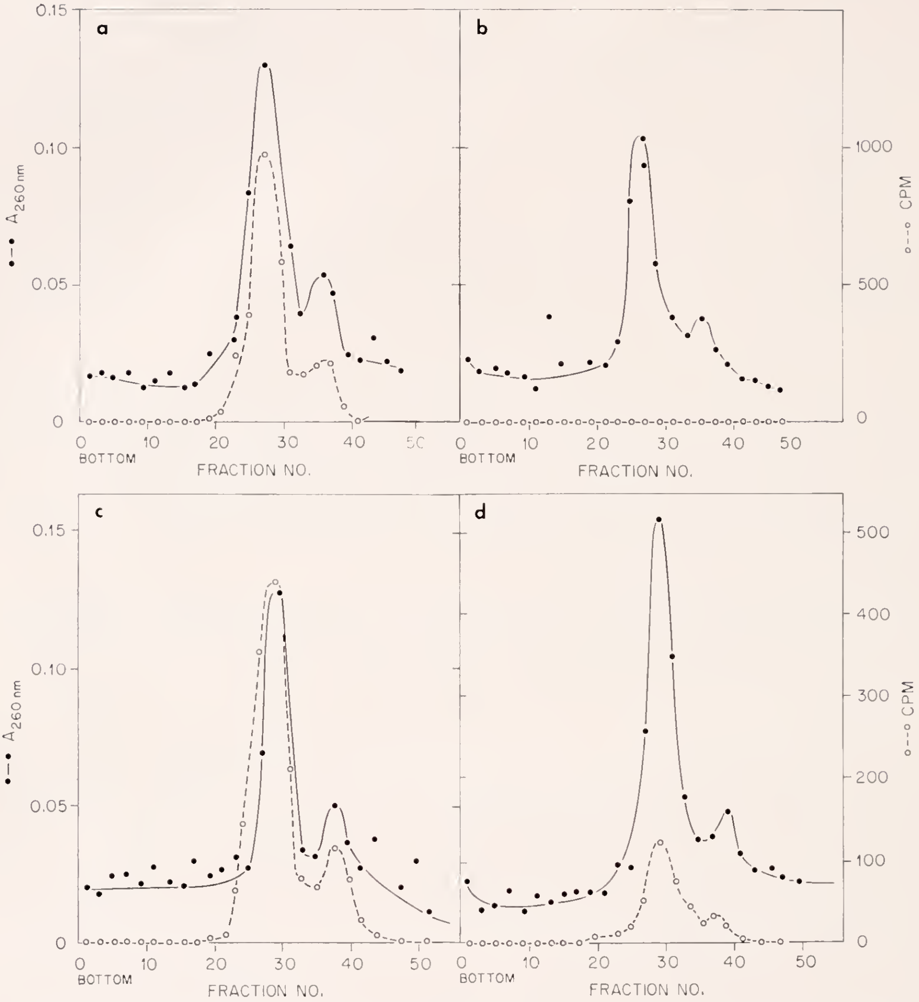

FIGURE 6. Preparative CsCl density gradients of DNA of regenerates from three animals at different stages of regeneration: (a) shows DNA isolated from four regenerates ($R = 14$) incubated with ^{3}H-thymidine as in Figure 4; (b), DNA isolated from bilaterally symmetrical partners to the regenerates in (a) autotomized 48 hr later. The closed circles joined by a solid line indicate absorbency at 260 nm; the open circles and dotted line indicate TCA-precipitable radioactivity; (c) and (d) were the same as (a) and (b) except that primary regenerates were removed early in Stage D_0 ($R = 8$).

shown above (Fig. 6c compared with Fig. 6a). DNA synthesis in their bilaterally symmetrical partners removed 48 hr later was approximately 25% the rate of those removed at time 0 (Fig. 6c and 6d; Table IV-B). This pattern of deceased rather than completely inhibited DNA synthesis corresponds to the observed decrease in the rate of growth of regenerates of similar size (*cf.* Fig. 2b). Larger primary regenerates ($R = 24$), autotomized on day 0, had approximately the same

TABLE IV

The effect of autotomy on DNA synthesis in remaining primary regenerates.

R†	SA of first set of regenerates SA(a)‡	SA of second set of regenerates SA(b)§	SA(b)/SA(a)
(A) Growth of primary regenerates stopped			
20	4.90	0.41	0.084
18	10.39	0.60	0.058
17	3.23	0.06	0.019
17	6.12	0.02	0.003
16	1.74	0.06	0.035
16	2.57	0.12	0.047
15	6.97	0.05	0.007
15	1.09	0.09	0.083
14	4.62	0.06	0.013
*13	14.05	0.86	0.061
*9	28.48	1.12	0.039
(B) Growth of primary regenerates decreased			
14	3.16	0.67	0.212
*13	10.13	2.07	0.204
12	7.28	2.04	0.280
*12	19.98	6.21	0.311
*12	3.44	1.89	0.550
9	6.04	0.56	0.093
8	1.56	0.47	0.301
(C) Growth of primary regenerates not affected			
24	0.15	0.18	1.20
20	0.70	0.69	0.99
18	1.40	1.41	1.01
*15	2.23	2.40	1.08
*14	4.90	5.09	1.04

* Animals without eyestalks.

† *R* = *R* value (see Materials and Methods).

‡ *SA* (*a*) = specific activity (cpm × $10^{-3}/\mu g$) DNA isolated from regenerates which had been removed on day 0, and then incubated *in vitro* 16 hr with ^3H-thymidine.

§ *SA* (*b*) = *as* SA(a), except regenerates removed on day 2.

rate of DNA synthesis as those removed from the same animal 48 hr later (data not shown). This corresponds to the data in Figure 2d which show that the growth of large primary regenerates is not inhibited by loss of their partners.

In several control experiments, both rates of DNA synthesis and growth patterns were determined on the same animals (data not shown). In one typical experiment, we found that three small regenerating limbs had a rapid synthetic rate; their three bilaterally symmetrical partners, autotomized 48 hr later, synthesized DNA at a decreased rate. Two regenerates were not removed; their rate of growth, followed for the ensuing ten days, decreased as had that of the primary regenerates of similar size previously described (Fig. 2b). Thus it was possible to observe in a single animal parallel inhibition of both growth and DNA synthesis. Again, re-regeneration of the missing legs occurred during the induced premolt pause.

Neither the rate of DNA synthesis (Table IV-C) nor the growth pattern (Fig. 2d) of very large regenerates was affected by the removal of one or more partial regenerates. These animals had apparently passed the critical period after which ecdysis can no longer be delayed (Skinner and Graham, 1972).

The differences between the rates of DNA synthesis of the partial regenerates removed on day 0 and those removed 48 hr later cannot be attributed to the normal size-associated decrease shown in Figure 5. In all experiments described, the size of the regenerates removed on day 0 was very similar to that of those removed 48 hr later. The time required for DNA synthesis to fall by 50% as a function of normal growth as in Figure 5 is on the order of a week. For DNA synthesis to be affected by as much as has been observed in Group A of Table IV requires more than a month if the regeneration is undisturbed. It is concluded that the decreased synthesis described in Table IV is not a function of the very small amount of growth that might occur in the 48 hr time interval between the two samples.

DNA synthesis in regenerates of animals without eyestalks

The data described thus far could be accounted for by the minimal hypothesis that the inhibition of growth and DNA synthesis after autotomy of partial regenerates is induced by secretion of the molt-inhibitory hormone, synthesized and stored in the crustacean eyestalk. To test this, experiments were performed on animals from which eyestalks had been removed. Data from regenerates of animals without eyestalks whose growth pattern was similar to that of regenerates of animals with eyestalks are indicated by an asterisk in Table IV. Growth and DNA synthesis of primary regenerates remaining after autotomy of their partners showed stage-dependent inhibition comparable to that observed in intact animals still capable of producing the molt inhibitory hormone.

In animals without eyestalks, the autotomy of slightly smaller partial regenerates ($R = 7$–10) produces complete inhibition of DNA synthesis equivalent to that caused by the loss of larger regenerates in animals with eyestalks. This smaller R value is comparable to that of animals whose DNA is depicted in Figures 6a and b since, as noted in the section *Growth of regenerates of animals with or without eyestalks,* at ecdysis primary regenerates of animals without eyestalks are smaller than those of animals with eystalks.

These experiments show that DNA synthesis and growth can be effectively blocked in the absence of molt-inhibitory hormone, indicating that an additional physiological inhibitory factor(s), not necessarily hormonal, is functional in these animals.

DISCUSSION

The coarse controls on molting are thought to be the interaction of two hormones: a molt inhibitory hormone (MIH), of the X-organ sinus gland complex, which predominates during the intermolt period; and a growth-promoting hormone, probably ecdysterone, that stimulates growth when present in a favorable concentration with respect to the antagonistic MIH. Although there must be many fine controls on the growth and differentiation of various tissues in the whole animal, the interaction of these two hormones has been thought to be primary in regulating "growth" *vs.* "non-growth". This simple picture is complicated by the present experiments in which growth inhibition and growth stimulation are simultaneously demonstrable in a single animal. In addition to the data described in the results above, apolysis and synthesis of a new exoskeleton are considerably delayed in animals whose premolt growth has been slowed by autotomy of partial regenerates (unpublished data).

Before discussing the present work on the limb regenerates and its relation to the work of others, the evidence for the functioning of MIH and ecdysterone or an ecdysterone-like hormone in Crustacea will be considered briefly. The role of MIH was suggested by experiments in which extirpation of X-organ or the X-organ sinus gland complex was effective in triggering molting (Passano, 1953). Rao (1965) isolated from the eyestalks of the crab, *Ocypode macrocera*, and partially characterized a peptide that delayed ecdysis when injected into eyestalkless animals. The role of the Y-organs was suggested by the fact that molting is prevented by the removal of Y-organs from the shore crab, *Carcinus maenas* (Echalier, 1954, 1959) and similarly, that after the Y-organ has been removed regenerates do not grow beyond basal plateau in the marsh crab, *Sesarma reticulatum* (Passano and Jyssum, 1963) and from the crab, *Pachygrapsus marmoratus* (Charmantier-Daures and Vernet, 1974). In a further set of experiments, the latter authors not only removed Y-organs but also eight pereiopods. In that case, 13 of 60 animals completed preparations for molt including complete regeneration of the missing limbs. Unfortunately, the authors did not indicate whether the animals, all of which died before ecdysis, were subjected to histological examination to determine if the Y-organs had been completely removed.

The possibility that ecdysterone is the active substance produced by the Y-organs was suggested by the isolation from green crabs of a substance that caused pupation of the fly, *Calliphora*, and was presumably ecdysterone (Karlson and Skinner, 1960). In addition, Horn and colleagues have isolated from green crabs a steroid with chromatographic and spectral properties similar to those of ecdysone isolated from insects (Hampshire and Horn, 1966; Horn, Middleton and Wunderlich, 1966). Commercially available ecdysterone is substantially less effective in Crustacea than in insects (Skinner and Graham, 1970) and may be highly toxic at doses that do not initiate molting (Lowe, Horn and Galbraith, 1968). The latter workers found that at lower doses, ecdysterone decreases the duration of the premolt period of crayfish that have already been stimulated to molt by eyestalk removal. Krishnakumaran and Schneiderman (1968) found it ineffective except at very high—although not pathological—levels in the amphipod, *Armadillidium*.

It is worth noting that although premolt growth of regenerates in crabs might require ecdysterone or an ecdysterone-like growth hormone, papilla formation and basal growth of regenerates do not show such dependence and occur in the absence of Y-organs (Passano and Jyssum, 1963) or even during intermolt when MIH is thought to predominate (Bliss, 1956; Hodge, 1958; Skinner, 1962).

The present paper is a continuation of earlier studies (Skinner and Graham, 1970, 1972) showing that the loss of more than four appendages is an adequate stimulus to molting preparations in intermolt specimens of *Gecarcinus.* This paper reports that the autotomy of only one (or more) of the ensuing primary regenerates may be effective in inhibiting further preparations for molt, *i.e.,* the continued growth of the remaining regenerates or exoskeleton formation; during the period of inhibition a second blastema forms at the autotomy plane of each missing primary regenerate. The magnitude of the inhibitory effect depends on the stage of the animal in its molting preparations. Prior to a certain critical time (late D_0 or early D_1), the growth of the remaining primary regenerates is slowed or stopped while the secondary regenerates "catch up". After the critical time (late D_1 to ecdysis) autotomy of partial regenerates does not influence the remaining regenerates or the duration of the molting period. It has long been known that limb regeneration in Crustacea is coordinated with molting. The second autotomy breaks the close tie between these growth events. The phenomena describd here have the effect of re-synchronizing the experimentally dissociated events of early premolt. Since regeneration is limited to this period and cannot occur during the long intermolt period, the biological adaptiveness of this re-synchrony is clear.

A similar interaction between the reactive (regenerating) tissue and the controlling system has been suggested in insects, where the controlling system is thought to be hormonal. Regeneration of an appendage or its imaginal disc delays ecdysis (O'Farrell and Stock, 1953, 1954; Stock and O'Farrell, 1954; Pohley, 1965; Madhavan and Schneiderman, 1969). Further, ecdysterone has been shown to be necessary for the regeneration of imaginal discs (Madhavan and Schneiderman, 1969) and has been implicated in the acceleration of DNA synthesis accompanying growth in insects (Krishnakumaran, Berry, Oberlander and Schneiderman, 1967; Oberlander, 1969).

Pohley (1961) found that the number of mitoses in a normal imaginal disc was decreased while a regenerate was being produced and postulated that the depletion or lack of a stimulatory substance (ecdysterone) could cause the inhibition of growth. Since the early phases of crustacean limb regeneration do not require the presence of ecdysterone (Passano and Jyssum, 1963), it is unlikely that the inhibition reported here could be caused by secondary regenerates selectively sequestering ecdysterone. Therefore, to account for the inhibition of premolt growth of primary regenerates by depletion of a stimulatory factor, a second factor would be required, such as an enzyme that specifically destroys ecdysterone. There is precedent for this suggestion. Between day 4 and 5 of the last larval instar in the hornworm, *Manduca sexta,* juvenile hormone rapidly disappears (Nijhout and Williams, 1974). A hormone-specific esterase has been postulated as the causative agent (Sanburg, Kramer, Kézly and Law, 1975).

The nature of the mechanisms controlling the events reported in this paper is not known. If the signal is hormonal, autotomy could cause the release of a blood-borne inhibitor. The inhibition of both growth and DNA synthesis in regenerates of animals without eyestalks, missing the putative source of MIH, leads to the conclusion that either another inhibitor is responsible or MIH can be produced (or stored and its release triggered by limb loss) elsewhere in the body.

In order to postulate that the controlling factor is neural, one must accept the constraint that the physiological response is determined to a large part by the target tissue. This conclusion is based on the following observations. First, assuming that the innervation of fully formed limbs is the same as that of their regenerates, cutting the *same* nerves stimulates preparations for a precocious ecydsis in one instance and inhibits premolt growth of primary regenerates and further preparations (other than the initiation of blastema formation of secondary regenerates) in a second. In both cases, the initial effect of the loss of five or more limbs or one or more partial regenerates is to cause a new cycle of regeneration of the lost limbs. Secondly, different *numbers* of nerves are involved in the two phenomena. The stimulation of precocious molts requires the loss of five or more limbs in *Gecarcinus*, the loss of only one partial regenerate inhibits growth and DNA synthesis in the remaining regenerates.

The long duration of the effect argues against a single nervous stimulus unless there are further consequences. In a possibly analogous case the bug, *Rhodnius prolixus,* undergoes molting preparations following the stimulation of the abdominal ganglia due to the distension of the body wall after a blood meal (Wigglesworth, 1934). It is thought that the nervous stimulus triggers the release of the prothoracicotropic hormone which, in turn, provokes the release of molting hormone (Wigglesworth, 1970).

The present demonstration of simultaneous inhibitory and stimulatory effects caused by the loss of primary regenerates emphasizes the fine and multifaceted control of the crustacean molt cycle.

NOTE ADDED IN PRESS

While this paper was in review, our attention was called to a publication by Tchernigovtzeff (1974). In general, that paper corroborates the results presented in this paper and our earlier reports (Holland and Skinner, 1974a, b). An apparent discrepancy is that in some cases Tchernigovtzeff failed to observe inhibition of growth of primary regenerates following the loss of a fully formed limb from animals missing eyestalks. The reasons for this discrepancy are not clear; we invariably found inhibition of both growth and DNA synthesis in all early premolt animals observed. The experimental protocols differed in several respects. In any case, Tchernigovtzeff's results and ours agree in demonstrating that growth inhibition and growth stimulation can occur simultaneously in individual animals. We conclude from these observations that there are finer controls than simply the ratio of two hormones in the general circulation.

SUMMARY

1. Following the loss of one or more primary regenerates from the land crab, *Gecarcinus lateralis,* before a critical stage of the molt cycle (probably D_1, early), secondary regenerates form to replace those lost.

2. Although loss of at least five limbs from an intermolt animal is required to initiate molting preparations (Skinner and Graham, 1972) loss of only one primary regenerate, prior to the critical time, is sufficient to inhibit these preparations.

3. Following the loss of one or more primary regenerates before a critical time, the rate of growth and the synthesis of DNA in other primary regenerates that remain *in situ* decrease or cease temporarily (10 to 14 days).

4. The precise stage of the animal in the molt cycle determines whether growth and/or DNA synthesis decrease or cease in the remaining primary regenerates. Loss of regenerates early in D_0 ($R = 7–10$) causes a decrease; loss of regenerates later in D_0 or early D_1 ($R = 10–17$) causes complete inhibition.

5. The duration of the premolt period is lengthened by the same time as that during which the early stages of re-regeneration of the missing regenerates occur.

6. Primary regenerates removed after the critical point in the premolt period are not re-regenerated before ecdysis; the premolt period is not extended and the animal emerges without a full complement of limbs.

7. Primary regenerates in animals without eyestalks respond similarly to those in animals with eyestalks. This excludes the possibility that the inhibition is caused by release of MIH from the sinus gland-X organ complex. Other physiological factors must be posulated.

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HEART AND SCAPHOGNATHITE RATE CHANGES IN A EURYHALINE CRAB, *CARCINUS MAENAS*, EXPOSED TO DILUTE ENVIRONMENTAL MEDIUM

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The mechanisms by which some crustaceans permanently or temporarily maintain their body fluids at osmotic or ionic concentrations different from those of their environment include the active transport of ions across the gills and other surfaces (Krogh, 1938; Green, Harsch, Barr, and Prosser, 1959; Shaw, 1961a), the limitation of diffusive permeability to water and ions of their surfaces (Smith, 1967, 1970; Shaw, 1961b; Rudy, 1967; Potts and Parry, 1964) and the control of the volume and/or ionic concentration of the urine (Shaw, 1961a; Green *et al.*, 1959). Some or all of the above processes appear to be controlled by hormonal mechanisms, especially in the case of crustaceans living in environments which vary in salinity. Although the physiological role of putative hormonal factors in hydromineral balance remains unclear in most cases, it is likely that endocrine factors from the eyestalk can effect the movement of sodium or water across the surface membranes in crabs and crayfish (Carlisle, 1955; Kamemoto, Kato, and Tucker, 1966; Kato and Kamemoto, 1969; Heit and Fingerman, 1975). Extracts of the thoracic ganglion and brain (which contain neurosecretory cell bodies) have likewise been found to alter the state of hydromineral balance or the movement of molecules when injected into whole animals (Kamemoto and Tullis, 1972) or when tested on isolated tissues (Mantel, 1968).

Tullis and Kamemoto (1974) have recently presented evidence for the presence in the thoracic ganglion and brain of the brackish water crab *Thalamita crenata* of two factors which appear to alter the permeability of surface tissues to water without altering the flux of sodium. A water-soluble component of small molecular weight decreases the water influx through the gills, while an acetone-soluble fraction increases the permeability of the gills, and perhaps of the gut as well. It is possible, however, that the changes in permeability to water do not reflect actual changes in the properties of the epithelium, since the rate at which water can move passively into or out of an animal not in osmotic equilibrium with its environment might be altered if the rate or pattern of circulation of the hemolymph in exposed surfaces (especially the gills) is altered, or if the rate of irrigation of the gills changes (Smith, 1970). Cornell (1973, 1974) has commented briefly on the possibility that changes in heart rate might account for an observed change in water flux in a stenohaline crab *Libinia emarginata*. In this paper we report on changes in heart rate in a euryhaline crab *Carcinus maenas* when transferred to dilute medium and discuss briefly the controlling mechanisms for the heart rate changes.

MATERIALS AND METHODS

Adult specimens of *Carcinus maenas* were obtained from the Marine Biological Laboratory, Woods Hole, Massachusetts, or were collected locally along the Connecticut shore. The animals, which weighed between 20 and 70 grams, were kept in circulating artificial sea water at 15° C in an Instant Ocean tank. Before use in an experiment an animal was placed in a shallow dish of sea water and allowed to equilibrate for at least 24 hours at 22–24° C, the temperature at which experiments were carried out. Ahsanullah and Newell (1971) have reported that there is very little thermal acclimation of heart rate in *C. maenas* over a period of 21 days, and all experiments were performed within this time after animals were removed from the tank. The fluids in equilibration and experimental chambers were vigorously aerated before and during the experiments, but the same results were obtained even if the chambers were not aerated during the course of the experiment.

Heart rate was monitored in freely moving crabs by the use of implanted electrocardiogram electrodes, which consisted of a short length of silver wire passed through a lucite block. The uninsulated end of the wire was inserted through a hole in the carapace, and the lucite block glued into place with Oneida Instant Weld adhesive. The best recordings were obtained when two electrodes were placed on the dorsal midline, one directly over the heart and one slightly anterior to it. Amplified signals were displayed on a chart recorder. About 75% of the implants yielded usable results.

The rate of scaphognathite beating was monitored in freely moving crabs by placing a cannula into the gill chamber through a hole in the dorsal surface. The cannula was attached to a pressure transducer, the output of which was amplified and displayed on a chart recorder. Most records were taken from the anterior portion of the epibranchial chamber.

Experiments were performed in smooth-sided chambers of white translucent plastic with snap-on lids. Smooth walls prevented the crabs from climbing out of the water, which they often attempted to do in response to being placed in highly diluted sea water. The use of translucent material minimized spontaneous heart stoppage and ventilation arrests which are often observed in response to visual stimuli. The chambers could be drained from the bottom, with fresh medium added rapidly from the top. All experiments were performed during natural daylight hours.

Artificial sea water was prepared by dissolving Instant Ocean sea salts in tap water. Dilutions were made by adding measured volumes of water to already mixed 100% SW, and are expressed as % SW. All solutions were vigorously aerated before being introduced into the chamber.

RESULTS

General characteristic of electrocardiogram records

Recorded heart rates varied markedly from animal to animal. Large crabs in general had slower rates than smaller crabs under the same conditions, as has been reported previously (Ahsanullah and Newell, 1971), and crabs that were monitored at lower temperatures had lower average frequencies (Florey and Kreibel, 1974). These overall trends were expected and were not extensively re-investigated, for it was found that individual variation of frequency of beat was considerable even after factors of size and ambient temperature were taken into account. The extent of the variability can be seen in Table I, where the five crabs numbered 1–5, all weighing between 30 and 32 grams, had heart rates at the beginning of their experimental runs which ranged from 101 to 184 beats/minute. In addition, there were often large differences in basal rate in the same crab from day to day (*e.g.*, for crabs 1, 5, 13 in Table I, compare the average starting rates on the control and experimental runs, which were performed on different days). The source of this variability is not clear. Apparently it is not simply due to the differences in general level of activity of the animal. Although rapid fluctuations in rate were often observed during periods of movement, the basal frequency normally returned rapidly to its previous level after the animal became quiet. In all of the records tabulated, readings were taken during prolonged periods during which there was no significant movement.

The heart frequency usually showed large, rapid and irregular changes when the medium was changed, and for several minutes thereafter. These fluctuations, which were not consistent in direction, were observed both when the salinity was changed and when the medium was simply renewed at the original salinity after the chamber was drained. Such fluctuations normally disappeared within a few minutes and only readings taken at least 20 minutes after a transfer were used in analyzing the results of these experiments.

Responses of the heart to dilute external medium

In this set of experiments, the medium was changed from 100% SW to 15% SW and then back to 100% SW. In control runs the same animals (on different days) were subjected to fresh 100% SW during the middle period. In each of 13 experimental runs of this type, the heart rate markedly increased when the animal was transferred to 15% SW. The average increase was 51 beats/minute, or 46%. These changes were observed whether transfer was accomplished by draining the tank completely and adding fresh 15% SW, or by adding the appropriate volume of tap water to 100% SW. When the animals were transferred from 100% SW to fresh 100% medium, only very small changes (increases or decreases) were observed, (averaging − 1 beat/minute, or − 1% from the basal level). The complete set of data is provided in Table I.

Results from a typical experiment are shown in Figure 1. The heart in most cases took about 30 minutes to reach its maximum, after which its rate stayed fairly constant for at least two hours. An increase was often noted within the first 10 minutes after transfer. Whether the response actually began before this point is not clear because of the fluctuations induced by the transfer. When the crab was returned to 100% SW after two hours in 15% SW, the heart rate gradually returned to about the level that it had shown initially in 100% SW. The time course of the return was quite variable. In some cases the frequency returned to the basal level by 18–20 minutes after transfer but more typically took over half an hour to come down.

TABLE I

Effects on heart rate of transferring Carcinus *from 100% SW to 15% SW (experimental) or from 100% SW to fresh 100% SW (control).*

Crab (Arranged by weight)	Sex	Weight (grams)	Experimental (100% SW → 15% SW)				Control (100% SW → 100% SW)			
			Average rate before (beats/min)	Average rate after	Net change	Percent change	Average rate before (beats/min)	Average rate after	Net change	Percent change
1	F	30	125	202	+77	+62%	197	188	−9	−5%
2	M	31	153	202	+49	+32%	159	157	−2	−1%
3	M	32	181	206	+25	+14%	194	195	+1	+1%
4	M	32	184	211	+27	+15%	161	162	+1	+1%
5	M	32	101	205	+104	+103%	190	196	+6	+3%
6	M	35	137	197	+60	+44%	148	149	+1	+1%
7	F	39	186	209	+23	+12%	191	186	−5	−2%
8	F	46	158	204	+46	+29%	159	156	−3	−2%
9	M	49	175	190	+15	+9%	175	177	+2	+1%
10	F	49	107	142	+35	+33%	74	68	−6	−8%
11	F	49	97	144	+47	+48%	109	123	+14	+13%
12	F	55	78	136	+58	+74%	104	95	−9	−9%
13	F	55	75	167	+92	+122%	38	36	−2	−5%
Means ±s.e.			135 ± 11	186 ± 8	+51 ± 8	46 ± 10%	146 ± 14	145 ± 14	−1 ± 2	−1 ± 2%

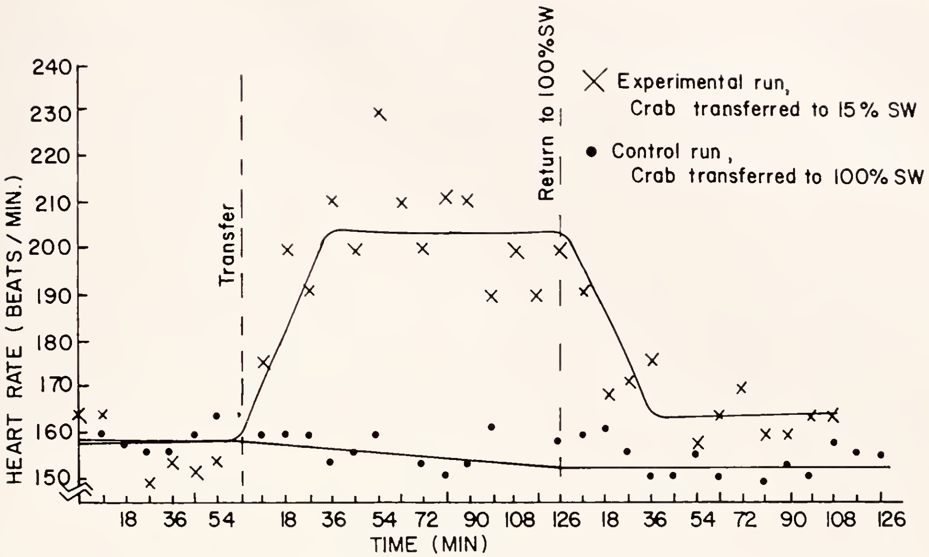

FIGURE 1. Heart rate of one *Carcinus maenas* during a control run (transfer to fresh 100% SW) and an experimental run (transfer to 15% SW). The two trials were run on different days. The results of this experiment are typical in terms of the increase of frequency (pattern, time course, and magnitude of the increase), but somewhat atypical in that both the control and experimental runs started at almost the same basal frequency.

In several of the experiments described below, in which smaller numbers of animals were used, the average basal heart rate before transfer to an experimental medium was considerably higher than that of the large group tabulated here. If the heart responses of all animals from Table I with a basal rate of 150 or higher

TABLE II

Effects on heart rate in Carcinus *of various experimental conditions.*

Experiment	External medium during test period	Conditions	Number of animals	Average rate before transfer (beats/min)	Average rate after transfer (beats/min)	Net change	Percent change
A	25% SW isosmotic with SW	sucrose added to maintain osmolarity	7	122 ± 10	152 ± 8	+30	+25%
B	low sodium	choline substituted for 75% of sodium	4	158 ± 25	147 ± 24	−11	−7%
C	low chloride	acetate substituted for 75% of chloride	4	174 ± 15	174 ± 13	0	0
D	15% SW	eyestalkless	5	146 ± 10	179 ± 9	+33	+23%
E	15% SW	all animals from Table 1 with a "before" rate of 150 or greater	6	173 ± 6	204 ± 3	+31	+18%

are analyzed as a separate group, a marked cardioacceleration is still observed after transfer to 15% SW (Table II, line E).

Relation between salinity and heart rate

By adding measured amounts of water to a known volume of 100% SW, the salinity in an experimental chamber was lowered in a number of steps. The crab was left in each solution for one hour, and the mean rate was calculated from the readings taken from 20 to 60 minutes after transfer. The averaged results from experiments on ten crabs are shown in Figure 2. From 100% SW down to about 75% SW, there was little or no cardioacceleration with decreasing salinity. Beyond this point, the rate increased as the salinity was decreased.

To insure that the order of exposure to different media did not account for the results, several animals were transferred directly from 100% SW to 15%, and then to one intermediate dilution (usually 40% or 50%) before being returned to 100% SW. In four animals tested, the heart rate was fastest in the most dilute medium and intermediate in the middle dilution.

Effect of osmotic concentration of the external medium on heart rate

To determine whether the cardioacceleration response is attributable to the lowered osmotic strength of dilute sea water, crabs were transferred to solutions

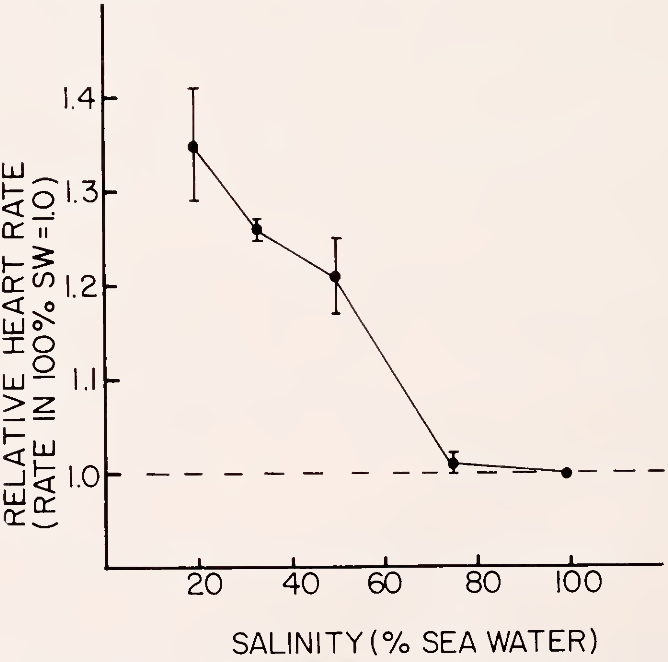

FIGURE 2. Heart response in *Carcinus maenas* as a function of salinity of the external medium. The vertical axis shows the ratio of the heart rate in diluted medium to that in 100% SW (mean value and standard error from experiments on ten animals). The nature of the curve is similar whether the salinity is reduced stepwise or the animal is transferred directly from sea water to the most dilute medium and then to intermediate salinities.

which were isosmotic with 100% SW but had only 25% of the ions found in normal sea water. Solutions were made isosmotic with sea water by adding an appropriate amount of sucrose to 25% SW. When transferred into the test medium, six of the seven animals tested showed a heart acceleration response similar to animals transferred to 25% SW (hyposomotic). The averaged results for all seven animals are shown in Table II, line A.

Effects of alterations of sodium and chloride concentrations on heart rate

The results cited above suggest that osmotic strength of the medium is not the parameter on which the cardioacceleratory response is based. It is possible that the crab perceives salinity changes by monitoring the concentration of one of the major ionic constituents of sea water. The effects of altering the concentration of specific ions was examined. One modified saline contained only 15% sodium but nearly normal chloride, and another 15% chloride but nearly normal sodium. The two modified media were prepared by adding 150 ml of 100% SW to each 850 ml of isotonic choline chloride in the first case, and 850 ml of isotonic sodium acetate in the second. The minor ionic constituents were therefore at 15% of their sea water concentrations in both modified media. Four crabs were tested in each altered sea water.

Neither ionic modification caused an acceleration of heart rate. When crabs were transferred from normal sea water to a medium with normal sodium but low chloride, the heart frequency was essentially unchanged (Table II, line C). In the normal chloride medium with low sodium, the rates in all four cases were slightly decreased (Table II, line B). Since no cardioacceleration was observed with either modification, and all other ions were low in both altered media, it is unlikely that the concentration of any specific ion in the external medium is the controlling parameter for the heart response.

Site of the receptors

In an attempt to localize the receptors for salinity changes a special chamber was constructed which allowed the front of the crab (with its mouthparts, antennules and antennae) to be exposed to one salinity, while the rear of the crab (including the gill chamber intake areas) could be exposed to different medium. Th crab was placed in the opening of an impermeable, tightly fitting rubber membrane (the neck of a rubber balloon) which was used to separate the two ends of a small lucite chamber. Each section of the chamber had an inflow and an outflow spigot which allowed independent perfusion of the two compartments. Both chambers were continuously perfused to prevent the accumulation of wastes or the depletion of oxygen. Since the incurrent openings of the gill cavity were in the rear chamber and the excurrent openings in the front, the animal tended to transfer the rear medium into the front chamber during normal pumping. To minimize the effects of such transfer, perfusion rates of the two chambers were rapid. It is likely, however, that particularly in the areas around the excurrent openings, the crab was exposed to a salinity intermediate between those stated for the front and rear chambers.

TABLE III

Effects on heart rate of independent perfusion of anterior and posterior ends of crabs.

Crab	Front in: 100% SW Back in: 100% SW		100% SW 15% SW	15% SW 15% SW	15% SW 100% SW
A	Rate	174 ± 2	188 ± 2	204 ± 2	188 ± 2
B	Rate	184 ± 2	181 ± 3	194 ± 1	184 ± 3

The experiments were performed by (1) first perfusing both chambers with 100% SW; (2) switching one chamber only to 15% SW; (3) perfusing both ends of the crab with 15% SW; (4) reversing the situation from that in step (2). The only firm conclusion that can be drawn from experiments on eight animals is that a maximal increase in heart rate occurs only if both ends of the crab are bathed in dilute medium. In some cases, slight increases in heart frequency were observed when one end or the other was in 15% SW (*e.g.*, crab A in Table III), while in other trials, no increase at all was seen unless both ends were in dilute medium (*e.g.*, crab B). The response to complete exposure to 15% SW was similar to that in the basic experiments described earlier despite the fact that the animals were immobilized by the membrane and had the claws removed.

In a second series of experiments suspected chemoreceptor structures were removed from several crabs. The antennules and antennae are known to contain chemoreceptors, which respond differentially to changes in external salinity in a number of crustaceans (van Weel and Christofferson, 1966; Lagerspetz and Mattila, 1961; Tazaki, 1975). Antennules were removed from four crabs. In all four animals the cardioacceleratory response to dilute saline persisted. Surgical removal of the antennae likewise did not eliminate the response in four animals, nor did the elimination of the chelae.

Effect of eyestalk removal

The neurosecretory center in the eyestalks of crustaceans is likely to exert some control over salt and water balance. In addition, some factor(s?) present in the X organ-sinus gland complex can apparently speed up isolated decapod hearts (Welsh, 1937; Knowles and Carlisle, 1956). Eyestalks were therefore removed from five crabs, to determine whether a hormonal factor from the neurosecretory complex might be involved in the excitatory response to dilute medium. In all five animals the heart frequency increased when the animal was placed in 15% SW, despite the absence of eyestalks (Table II, line D).

Effects of dilute medium on scaphognathite rate

Ten crabs with a gill chamber cannulated were transferred from 100% SW to 15% SW for one to two hours and then returned to sea water. In six of these animals the scaphognathite rate was markedly *decreased* when the animal was in dilute medium. The scaphognathite not only beat more slowly, but in several cases, intermittantly stopped beating for periods of up to several seconds (Figure 3). In the other four animals, no consistent changes in rate in either direction were noted. None of the animals transferred to fresh 100% medium showed consistent changes in scaphognathite rate.

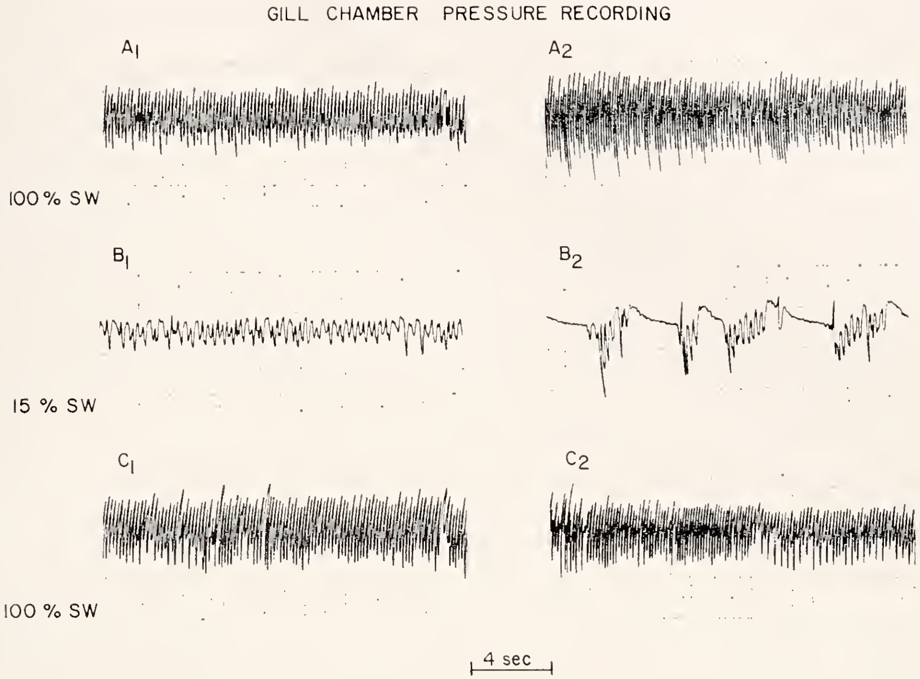

FIGURE 3. Pressure transducer records from the epibranchial cavity of *Carcinus* during exposure to normal and diluted sea water. A), pretest in 100% SW; B), exposure to 15% SW; C), retest in 100% SW. In each case, the right tracing was taken later than that on the left. The spontaneous stoppages of beating seen in 15% SW (B_2) were almost never seen in 100% SW, although arrests can be induced by visual, tactile, or auditory disturbances.

DISCUSSION

A marked increase in heart rate is observed in the euryhaline decapod *Carcinus maenas* when the animal is transferred from 100% SW to a more dilute medium. Cardioacceleration is not observed until the environmental medium falls below 75% SW. *Carcinus* is an osmoconformer between 100% and 75% SW and begins to hyperregulate only when the external salinity falls below this level (Shaw, 1961a). It therefore seems likely that cardioacceleration is part of an adaptive regulatory response to dilute medium.

Similar heart responses have been reported previously in representatives of two other groups of crustaceans, the euryhaline amphipod *Gammarus duebeni* (Kinne, 1952), and in the euryhaline shrimp *Crangon crangon* (Spaargaren, 1973). In the stenohaline crab *Libinia emarginata,* however, contrasting results have been reported. Cornell (1973, 1974) has reported a decrease in heart rate when individuals of this species are transferred from 100% SW to 80% SW, which is near the lower limits of its tolerance. Cornell has hypothesized that a decrease in heart rate might account for the decrease in water permeability of the body surface that is observed in some crabs when they are placed in a medium which differs in osmotic concentration from that of their body fluids. If a decline in the heart rate results in a corresponding decrease in the flow of hemolymph through the gills, thus reducing the average gradient for the inward movement of water, a decrease in water flux could result without a real change in the properties of the gill epithelium. Our results suggest that in euryhaline crustaceans different mechanisms operate.

Interpretation of the adaptive value of an increase in heart rate depends on a knowledge of the cardiac output of the heart. It is generally assumed that as the frequency of beat increases cardiac output also increases. Florey and Kreibel (1974), have suggested from studies in *Cancer magister*, that increased heart rate might, under some conditions, lead to a decrease in cardiac output due to incomplete filling of the heart at each beat. This conclusion, based on calculations from respiratory data of Johansen, Lenfant and Mecklenberg, (1970) depends on the extremely large temperature coefficient of the p50 of crab hemocyanin. If, in *Carcinus* held at a constant temperature, heart rate increase results in decreased cardiac output, the correspondingly lower flow of hemolymph through the gills might directly account for the observed decline in "water permeability". If, on the other hand, cardiac output increases with increased rate, the adaptive value of the observed response might be related to one or more of several physiological factors involved in hyperosmotic regulation: 1), an increased flow of hemolymph through the gills might be a mechanism to facilitate oxygen uptake, which increases markedly in *Carcinus* in dilute medium (Potts and Parry, 1964); 2), an increased cardiac output could lead to more effective dispersion of metabolic substrates and hormones involved in regulation; and/or 3) the volume of urine produced by *Carcinus* increases by a factor of about ten when the animal is transferred from 100% SW to 40% SW (Shaw, 1961a). Urine is apparently formed by a filtration mechanism (Binns, 1969; Lockwood and Inman, 1973; Riegel, Lockwood, Norfold, Bulleid, and Taylor, 1974), and an increase in heart output might enhance filtration. A more detailed discussion of the possible significance of the heart rate increase awaits the availability of detailed information on cardiac output as a function of heart rate in *Carcinus* at constant temperature.

We have not yet delineated the pathways by which salinity changes in the external medium exert a control over heart rate. Our attempts to localize receptors for the salinity change have yielded inclusive results. It is possible that internal salinity receptors are involved, but we do not consider this likely. The internal osmotic concentration of *Carcinus* falls only very slowly (Margaria, 1931; Shaw, 1961a) and reaches its lowest levels only after 12–15 hours of exposure of the animal to dilute medium. After one to two hours of exposure to 33% SW or 25% SW, the internal concentration drops by less than 10%, and is still far above the final level reached. In addition, the rate of fall of internal concentrations appears to be similar when the animal is exposed to a range of external salinities below 50% (Margaria, 1931). The heart response reported here levels off after 10–30 minutes exposure to dilute medium despite the fact that the internal salinity is still falling. In our experiments where animals were transferred from 100% SW to 15% SW for a short period, and then placed at an intermediate salinity (40% or 50% SW), the heart rate was faster in 15% SW than in the intermediate salinity. Since the internal salinity is continuously falling throughout the course of this experiment, it appears that the heart rate is more closely linked to an external factor than to any internal one.

The observation that a maximal response is observed only when both ends of the animal are exposed to dilute medium might imply that cooperative interaction between receptors in different areas (*e.g.*, anterior appendages and gill chambers) is involved. Alternatively, receptors in the region of the mouthparts or the exhalant openings of the gill chambers may mediate the response. Salinity-sensitive cells in either of these areas might be influenced by the solutions in both chambers in our experimental design, since fluid from the rear chamber is pumped forward by the animal over these sites. The antennules, antennae and chelae have been eliminated as the only receptor sites mediating the response (despite the presence of salinity receptors on some of these appendages) since their ablation does not alter the reults of transfer to 15% SW.

The output pathway of the heart regulatory system in the context of osmotic regulation is likewise not yet determined. A direct effect of dilution of the internal fluids on heart function appears to be ruled out for the same reason that internal receptors were eliminated from consideration. The heart is subject to excitatory control by regulatory axons which synapse on the cardiac ganglion cells, and possibly by hormones from several sources (Maynard, 1960; Knowles and Carlisle, 1956). Cardioexcitor factors, which can be released from neurosecretory endings into the bathing medium and are presumably hormonal in nature, are found in the pericardial organs and in the sinus gland-X organ system. The physiological conditions under which these possible cardioexcitor hormones are released into the circulation in intact animals are almost completely unknown. The sinus gland has been eliminated as a source of cardioexcitor hormone in osmoregulatory adjustments, since extirpation of the entire eyestalk does not abolish the response. We are currently investigating the possible role of pericardial organs as part of the pathway by which external salinity changes cause changes in heart rate. There have been reports of differences in relative weights of pericardial organs in euryhaline as compared to stenohaline species of crabs (Maynard, 1961), but no information has been published to date that indicates differences in cardioexcitor content or secretion rates.

The striking similarities between the control of the cardioacceleratory response reported here and the permeability changes described by Smith (1967, 1970) and Capen (1972) in the euryhaline *Rhithropanopeus* and *Carcinus* are worth noting. Smith found that no significant permeability change occurs in *Carcinus* until the medium drops below 75% SW; there is also no cardioacceleration down to this level. In *Rhithropanopeus*, water permeability decreases in dilute sea water made isosomotic to 100% SW by the addition of sucrose while no permeability changes are observed in low Na^+ or low Cl^- sea waters; the heart rate changes described here show the same osmotic and ionic dependencies. Removal of antennules does not effect permeability changes in *Rhithropanopeus* nor does it effect the cardioacceleratory response in *Carcinus*. The heart response appears to reach its peak more rapidly than does the water permeability change. This may in part be due to the nature of the recording techniques, which allow an analysis of events occurring over a shorter time course in the case of heart rate, but it may also represent a real difference in the output end of the system. Capen's experiments do not distinguish between hormonal and nervous nature of the output, nor do ours, but the similarities of the two responses suggest that the heart rate changes and the permeability changes are parts of the same adaptive mechanisms which are induced to cope with a state of hydromineral imbalance.

A sharp decrease in scaphognathite rate occurred in more than half of the crabs transferred to a dilute medium. The variability in the time course of the drop in frequency in responding animals was much greater than it was for the heart rate change; in some cases the scaphognathite rate dropped off within a minute after transfer, but in others the rate was maintained at its previous level for half an hour or more before starting to decline. If a decrease in scaphognathite rate is a mechanism for limiting the exchange of material across the gills, it is not clear why the remaining animals gave no response at all. Arudpragasam and Naylor (1964a, b) have argued that the periodic reversals of scaphognathite beating may be functionally important in ventilation, and that the frequency of beating of the appendage during normal forward pumping may not be an accurate parameter of the potential for exchange. These conclusions have been challenged on the basis of careful measurements of pO_2 and hydrostatic pressure changes in different regions of the gill chamber during reversal (Hughes, Knights, and Scammel, 1969). In our experiments there were no consistent changes in the frequency of reversals of the pump, either in animals which showed a marked decline in the rate of forward beating, or in animals which retained the normal rapid forward pumping in dilute medium.

There have been numerous previous reports of coordinated changes in heart and scaphognathite rate in several crustaceans. Low oxygen content in the medium induces a simultaneous slowdown in lobsters (McMahon and Wilkens, 1972). In *Carcinus* the same treatment slows the heart and has little effect on the frequency of forward pumping, but may alter the nature of reversals of the scaphognathite (Taylor, Butler, and Sherlock, 1973). A variety of tactile and chemical stimuli can cause reflex changes in the two systems in the same direction [CO_2, N_2, NaCl and sugars cause slowdown or stoppages of both in the crayfish (Larimer, 1964; Ashby and Larimer, 1965)]. Conversely, jets of water cause a decrease in heart rate and an increase in ventilation in crayfish (Larimer, 1964). Wilkens, Wilkens, and McMahon (1974) have reported the presence of interneurons in the connectives of the crab *Cancer magister* which alter the function of both systems. In addition, factor(s?) contained in a crude extract of pericardial organs speed up scaphognathite rate as well as heart rate (Berlind, unpublished). Very few naturally occurring stiumli cause an increase in heart rate. It is clear that, while heart and ventilation responses are often coupled, they are to some degree subject to independent control or can change in opposite directions in response to the same stimulus. It is of interest with regard to this point that 5 hydroxytryptamine, a suspected neurotransmitter or neurohormone in crabs speeds up the heart but slows the scaphognathite (Berlind, unpublished). The control mechanisms which account for the different responses or heart and scaphognathite in the context of osmoregulation remain to be determined.

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SUMMARY

When the euryhaline decapod *Carcinus maenas* is transferred from sea water to a medium more dilute than 75% sea water, the frequency of heart beat increases. The cardioacceleratory response is probably mediated by external rather than internal receptors, and the parameter that is sensed in neither the total osmotic strength of the medium, nor the concentration of any single ion. Although it is not yet determined whether the heart responds to a hormonal or nervous signal, it is likely that the control mechanisms are similar to those which mediate the water permeability changes in *Carcinus* and other euryhaline crabs.

In somewhat more than half of the crabs tested, the frequency of scaphognathite beating is markedly reduced when the animal is exposed to dilute external medium.

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THE LARVAL DEVELOPMENT AND METAMORPHOSIS OF THE PEDUNCULATE BARNACLE *OCTOLASMIS MÜLLERI* (COKER, 1902) REARED IN THE LABORATORY[1]

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Octolasmis mülleri (Coker, 1902) is a small pedunculate barnacle found attached to the gills of the blue crab, *Callinectes sapidus* and other decapod hosts (Walker, 1974). First described from Beaufort, North Carolina, by Coker (1902), *O. mülleri* is perhaps synonymous to the cosmopolitan *Octolasmis lowei* (Nilsson-Cantell, 1927; Causey, 1961) or a local race or subspecies (Pilsbry, 1953) of a shallow-water *O. lowei*-series (Newman, 1967). In the western Atlantic its known range extends southward from the Chesapeake Bay (Van Engel, 1972) through the Gulf of Mexico and Caribbean Sea to Brazil (Lacombe, personal communication). Although Coker (1902) noted an increase in barnacle numbers in late summer, the breeding season is unknown.

The complete larval deveolpment for this species or other members of the family Poecilasmatidae has not been described. The first and second naupliar stages and cyprid of *O. mülleri* were described by Coker (1902) but attempts to rear larvae failed. Of the pedunculate barnacles in general, complete larval descriptions exist only for *Scalpellum scalpellum* L. (Kaufmann, 1965), *Pollicipes mitella* L. (Yasugi, 1937), *Pollicipes spinosus* Quoy and Gamard (Batham, 1945b), *Pollicipes polymerus* Sowerby (Lewis, 1975), *Ibla idiotica* Batham (Batham, 1945a), *Ibla quadrivalvis* Cuv. (Anderson, 1965), *Lepas fascicularis* Ellis and Solander (Willemos-Suhm, 1876; Bainbridge and Roskell, 1966) and *Lepas pectinata* L. and *Lepas anatifera* Spenglner (Moyse, personal communication).

The present study was undertaken to determine the breeding season of *O. mülleri* in a South Carolina estuary, to describe the life stages of the species through laboratory rearing and to establish criteria for staging of larvae in future experiments.

METHODS

Using wire mesh crab pots, blue crabs were collected at two to four week intervals at North Inlet, South Carolina from June 1974 to October 1975. Crab gills were examined for *O. mülleri*, the total number of barnacles and the number gravid (egg mass visible in the mantle cavity) being noted. During the summer and fall (July–November), gravid barnacles were teased off the gills and isolated in 35‰ sea water at room temperature (24–29° C).

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Released stage I nauplii were placed in fresh sea water and following molting to stage II, larvae were transferred to 8 cm Carolina culture dishes, 50–100 larvae per dish. For determination of development time, active larvae were transferred to 5.5 cm dishes, five larvae per dish. The algae *Monochrysis* sp., *Isochrysis galbana, Pseudoisochrysis paradoxa, Tetraselmis suecia,* or *Dunaliella tertiolecta* were added to individual cultures. Plant cells and sea water were changed at two-day intervals. Based on original results, fifteen additional larvae were reared on a mixture of *Monochrysis* and *Tetraselmis,* five larvae per 5.5 cm dish. All cultures were maintained at 30‰ and room temperature.

Dead larvae and exuviae were preserved in 70% ethanol. Drawings were made from freshly sacrificed larvae slightly flattened by coverslip pressure using camera lucida. Measurements were made with an ocular micrometer on unflattened preserved larvae. Total length was measured from the mid-anterior carapace edge to the dorsal spine tip. Carapace width was measured at the widest part of the carapace. Carapace length (stages, V, VI) was measured from the mid-anterior to mid-posterior carapace edges.

Some aspects of the external morphology of settlement and metamorphosis of *O. mülleri* larvae were observed by exposing a newly molted blue crab to ten reared cyprid larvae. The crab gills were examined 16 hours later and gill platelets with attached larvae were removed and placed in 35‰ sea water.

RESULTS

O. mülleri were present on blue crabs every month from June 1974 to October 1975. Incidence on crabs was variable and is presently being studied in detail. Gravid barnacles were noted every month, the percent of gravid individuals varying from below 10% during February–March to above 40% during August–November. Cyprid larvae (attached to the gills), were found during July–October. Up to 70 larvae were found per crab, incidence being greatest in September and early October.

Larvae reared in the laboratory pass through six free-swimming naupliar stages and one cyprid stage. In the small cultures, *Dunaliella* produced 100% mortality of barnacle larvae within two days. All other algal species supported some larvae development: *Tetraselmis,* stage III; *Pseudoisochrysis,* stage III; *Isochrysis,* stage V; and *Monochrysis,* stage VI. Only the mixture of *Monochrysis* and

TABLE I

Mortality (%) and earliest day of molt for each stage of reared larvae of Octolasmis mülleri.

Food	Stage II initial number	II		III		IV		V		VI		Cyprid	
		%	day	%	day	%	day	%	day	%	day	%	day
Monochrysis	30	17	1	32	6	41	10	40	14	100	18	—	—
Monochrysis/ Tetraselmis	15	0	1	0	4	0	6	0	7	0	9	7	15

Tetraselmis allowed complete development to cyprid (Table I). For *Monochrysis,* stage VI larvae developed in 18–22 days; for *Monochrysis/Tetraselmis,* larvae developed in 9–10 days. In the latter culture, development time to cyprid ranged from 14 to 18 days.

Larvae in the large cultures became tangled in clumps of three to ten individuals, resulting in substantial initial mortality. Otherwise the effects of different algae species were similar to small culture results.

Larvae

The most significant characters for naupliar and cyprid stages follow.

Stage I. (Fig. 1, I). Accurately described by Coker (1902), this stage is characterized by curved frontolateral horns projecting caudolaterally and short undeveloped caudal spine and abdominal process. Molting to stage II begins almost immediately after release from the mantle cavity.

Stage II. (Fig. 1, II). Present findings vary somewhat on setule arrangements indicated by Coker (1902) (Fig. 2). The nauplius assumes a form distinct from reported balanomorph larvae. A long barbed caudal spine and a shorter barbed abdominal process are present. No distinct lateral spines exist on the carapace. Frontal filaments are present in this and all subsequent naupliar stages.

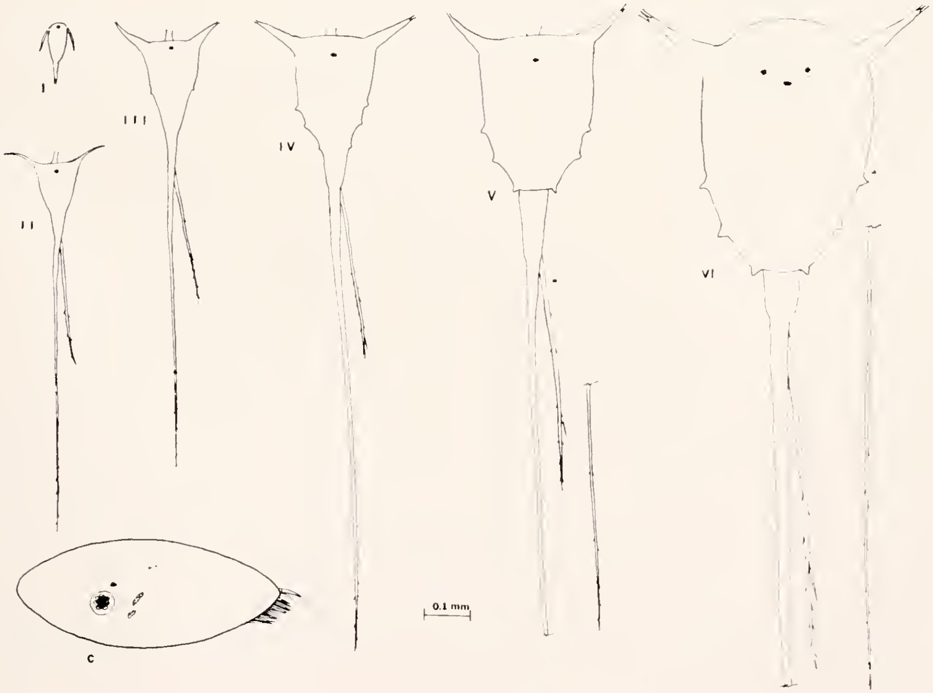

FIGURE 1. Carapace outlines of the six naupliar stages (I–VI) and cyprid (C) of *Octolasmis mülleri.*

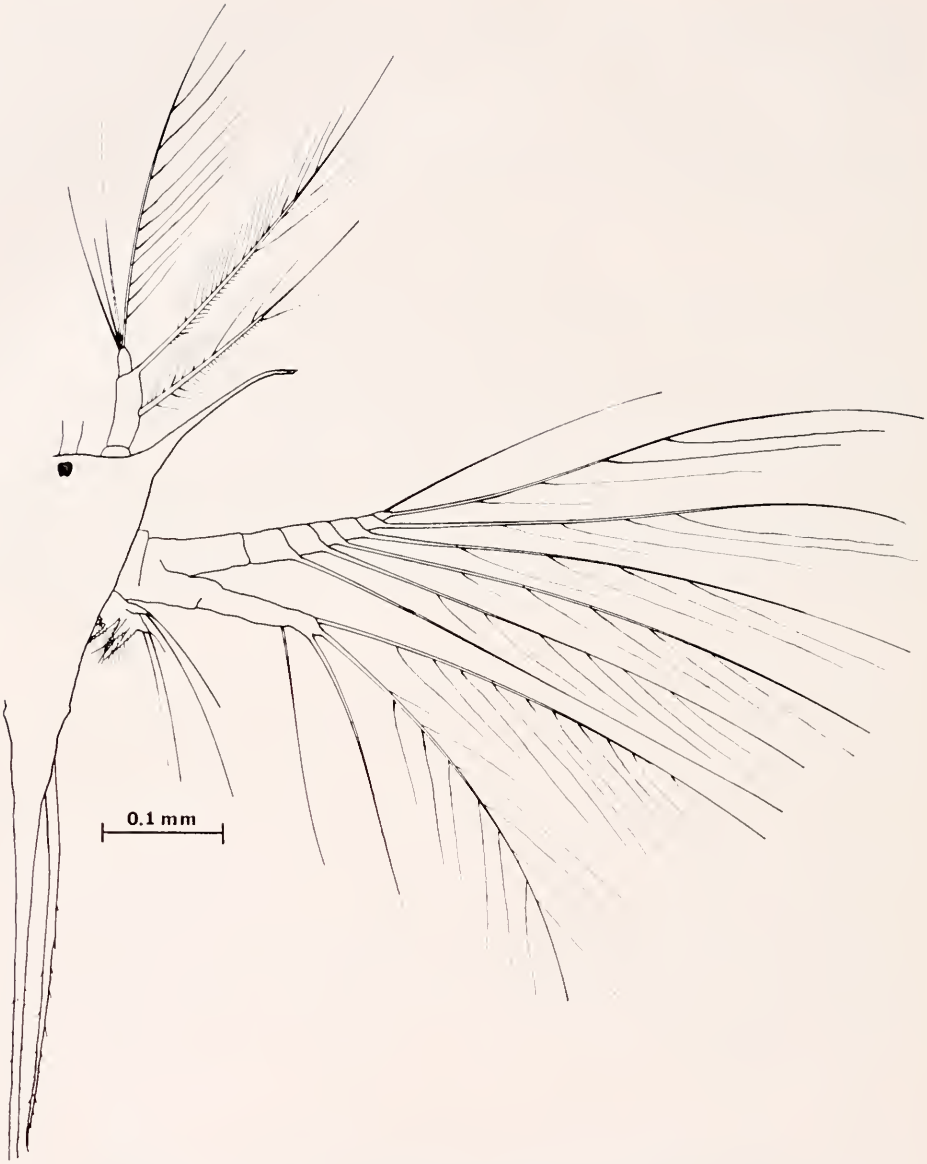

FIGURE 2. Dorsal view of the stage II naupli of *Octolasmis mülleri* showing complete setation of appendages.

The frontolateral horns project forward and have a plain tip (Fig. 3A). The single-lobed labrum tapers to a small rounded tip, the form remaining constant in all stages (Fig. 4). Setae line the distal portion.

Stage III. (Fig. 1, III). The frontolateral horn tip is trifurcate with tufts of fine setae and one minute setose seta extending from the central core. Apparently

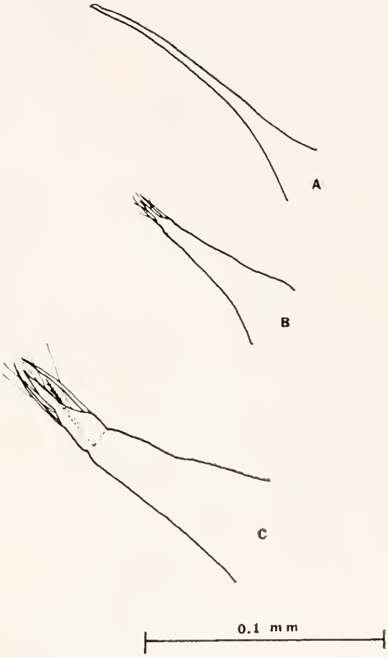

FIGURE 3. Ventral view of the frontolateral horns of stage II (A), stage III (B) and stage VI (C) nauplii. The number of fine setae has been reduced for graphic clarity.

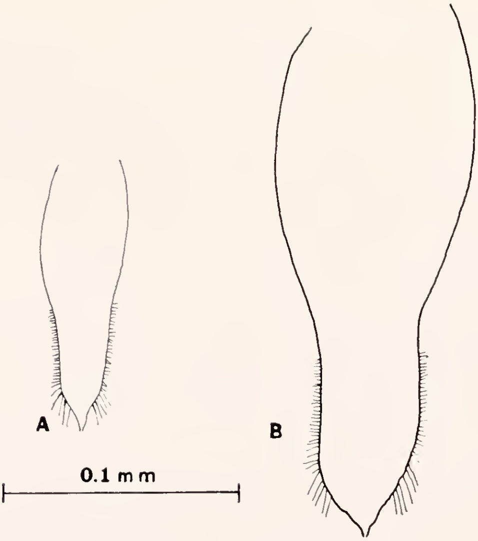

FIGURE 4. Ventral view of the labrum of stage II (A) and stage VI (B) nauplii.

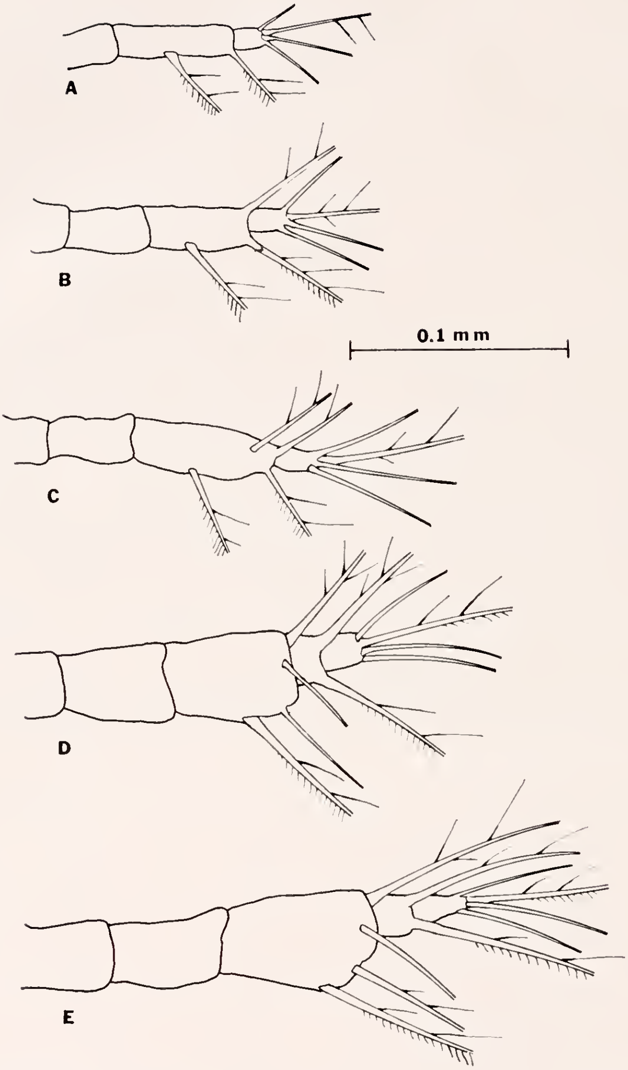

FIGURE 5. Antennules of stage II (A), stage III (B), stage IV (C), stage V (D) and stage VI (E) nauplii. A series of short lines indicates a bristled shaft (see text).

sensory in nature, this structure is present in stages III–VI (Fig. 3). One pair of lateral spines is distinct on the carapace. A pre-axial seta is found on the antenna (Fig. 6B).

Stage IV. (Fig. 1, IV). The carapace bears three pairs of lateral spines. The abdominal process has one distinct spine at its dorsal portion.

Stage V. (Fig. 1, V). The posterior border of the carapace is clearly defined and the lateral spines are proportionally larger. A swelling of the antennule third segment is evident (Fig. 6D).

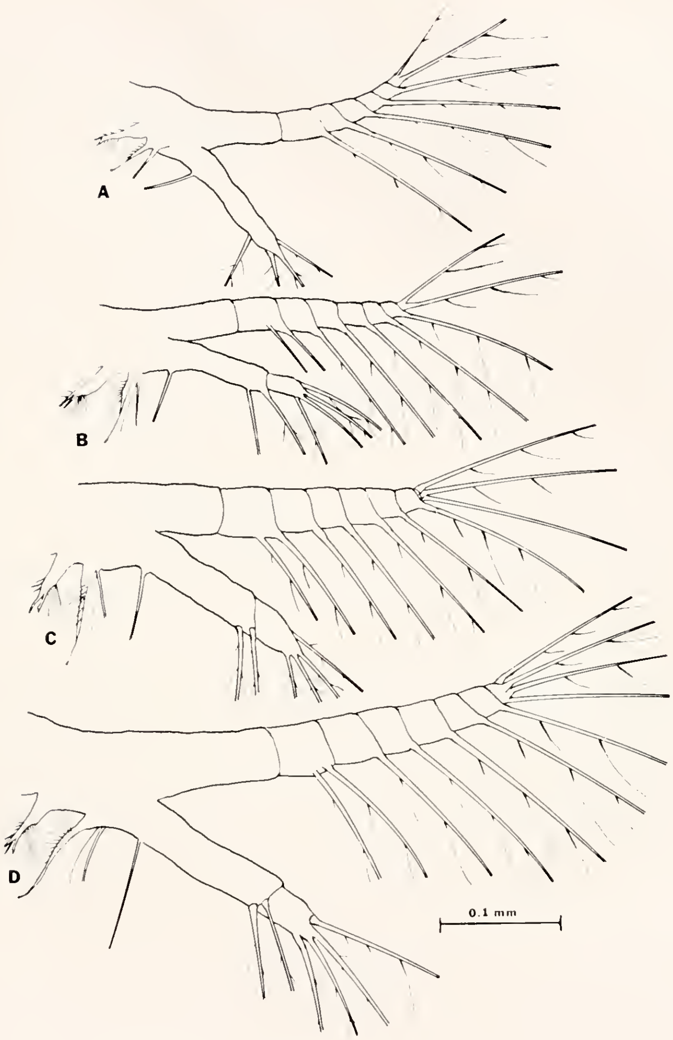

FIGURE 6. Antennae of stage III (A), stage IV (B), stage V (C) and stage VI (D) nauplii.

Stage VI. (Fig. 1, VI). The carapace broadens to an oval shape, obscuring dorsally the mandibles and frontal filaments. No paired eyespots are evident initially but become distinct within two to three days. Thoracic limb outlines are visible through the cuticle.

Cyprid. (Fig. 1, C). The carapace form is typical of most described cyprid larvae. In live specimens the cyprid is transparent with an amber tinge. A distinct orange pigmented area is present slightly posterior to the paired eyes.

Setation becomes more elaborate with successive molts (Figs. 5–7). Relative to balanid larval appendages, the antennule and antenna of *O. mülleri* nauplii are

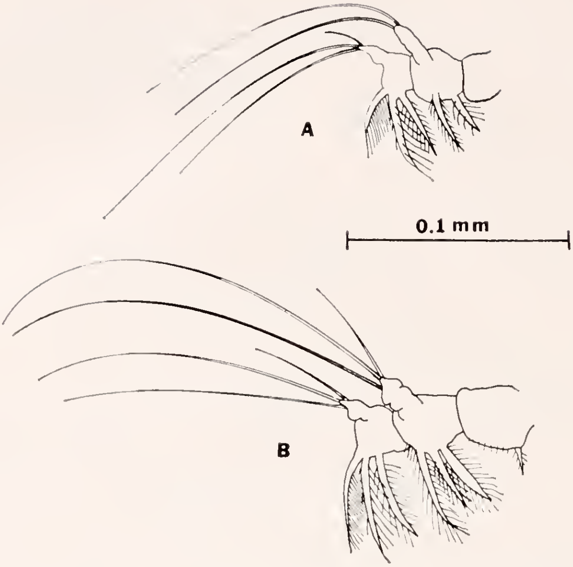

Figure 7. Mandibles of stage II (A) and stage V (B) nauplii.

elongate while the mandible is small with a very simple and indistinctly segmented exopodite (Fig. 7). Setae and setules are generally long and very delicate. Rather than the typical arrangement of hairlike setules coming off opposite sides of the setal shaft, the plumose setae of *O. mülleri* often have setules concentrated on one side of the shaft. This is particularly evident on the antennal exopodite. The antennule bears one (stage II) to three (stages IV–VI) setae with relatively thick shafts lined with short bristle-like setules and numerous closely spaced long setules (Fig. 2). These appear to serve as fine filtering devices. The setation formula of Newman (1965) as modified by Sandison (1967) is given in Table II. No major variations in setation were observed in this study.

The triangular carapace is smooth and transparent with no dorsal spines. A distinct pair of marginal spines first appears at stage III. These increase to three

Table II

Setation formula for nauplii of Octolasmis mülleri.

Stage	Antennule	Antenna		Mandible	
		Exopodite	Endopodite	Exopodite	Endopodite
6	P P SPSS P S P P	3PS 7P	4P 2P S SSC G	3S 3S	CPP PPP G
5	P P SPSS P S S P	2PS 7P	4P 2P S SSC G	3S 3S	CPP PPP G
4	P P SPSS P P	2P 5P2S	3PSPS S SSC G	2S 3S	CPP PPP G
3	P SPSS P P	2P 5P	3P P S S C G	2S 3S	CPP PPP G
2	SPSS P P	SP 4PS	2PSS S S C G	2S 3S	CPP PP
1	SSSS S S	SS SSS	3S S S G	2S 2S	SSS SS

TABLE III

Measurements of larval stages of Octolasmis mülleri *reared under laboratory conditions.*

Stage	Number measured	Carapace				Total length (mm)	
		Width (mm)		Length (mm)			
		Range	x̄	Range	x̄	Range	x̄
II	8	(0.10–0.11)	0.107	—	—	(0.79–0.85)	0.814
III	10	(0.12–0.14)	0.125	—	—	(0.92–1.11)	1.004
IV	9	(0.16–0.18)	0.173	—	—	(1.18–1.34)	1.270
V	6	(0.23–0.27)	0.247	(0.32–0.36)	0.343	(1.67–1.82)	1.735
VI	9	(0.36–0.39)	0.378	(0.52–0.55)	0.535	(2.54–2.78)	2.659
cyprid-reared	10	(0.21–0.26)	0.223	(0.54–0.59)	0.572	—	—
cyprid-"wild"	4	(0.21–0.23)	0.222	(0.54–0.57)	0.567	—	—

pairs at stage IV. A well-defined posterior carapace border develops only after the fourth molt. The marginal spines are often less developed on one side relative to the other and at times one or more will be missing entirely. The carapace size remains relatively constant per stage with a distinct size increase at each molt (Table III).

Reared cyprids were uniform in shape and nearly identical to "wild" specimens in both size (Table III) and pigmentation. A distinct orange pigment is usually restricted to one or two compact areas between the compound eyes and natatory appendages. The color remains evident through metamorphosis and perhaps is similar to the yellow cells of *Balanus balanoides* cyprids (Walley, 1969). The bright orange pigment however is distinct from any coloration observed in cyprids of *Balanus eburneus* and *Chelonibia patula* reared in this laboratory.

A sequence of abdominal spines similar to that of Balanidae (Moyse, 1961) or *Lepas fascicularis* (Bainbridge and Roskel, 1966) does not occur in *O. mülleri*. A paired row of small spines is sometimes seen on the abdominal process of stage II nauplii (Coker, 1902) but is not evident in later stages. The abdominal process is covered with numerous spinules and in stages IV–VI has a single spine near its distal end. The lesser barbed caudal spine is longer than the abdominal process in all stages.

The tapered single-lobed labrum of *O. mülleri* is distinct from the broad, flat-edged labrums of *Lepas fascicularis* (Bainbridge and Roskell, 1966) or *Pollicipes mitella* (Yasugi, 1937) and *Pollicipes polymerus* (Lewis, 1975). This form may be characteristic for the genus but comparative descriptions are needed.

Cyprids introduced to a newly molted blue crab were rapidly drawn into the gill chamber. Of ten initial larvae, six were found attached to the gills 16 hours later. The exact times of attachment are unknown but in each both antennules of the cyprid were cemented to a single gill platelet (Fig. 8A). After removal from the gill, four cyprids continued metamorphosis to adults. At initial recovery the peduncle outline was visible through the cyprid carapace (Fig. 8A). The capitulum outline became progressively more distinct while internal areas turned

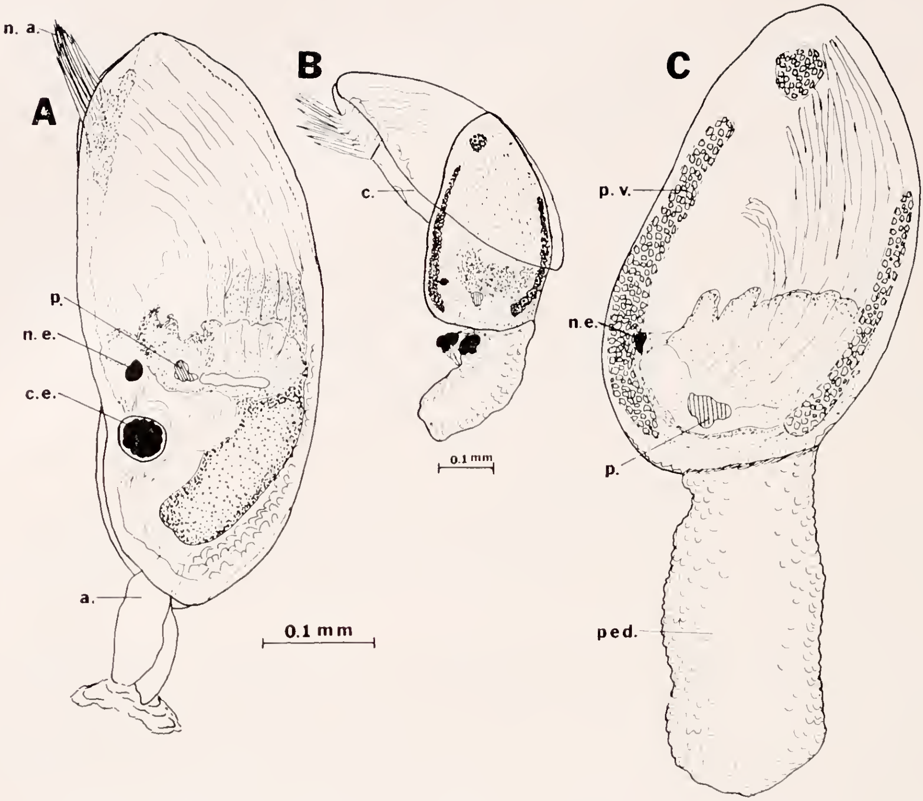

FIGURE 8. A. Cyprid of *Octolasmis mülleri* recovered from blue crab gill 16 hours after exposure. Prominent features include the cemented antennules (a.), compound eye (c.e.), natatory appendages (n.a.), nauplius eye (n.e.) and orange pigment spot (p.). B. By 36 hours after recovery the cyprid carapace (c.) is nearly shed. C. The newly emerged juvenile barnacle still retains the nauplius eye (n.e.) and pigment spot (p.). Primordial valves (p.v.) and expanded peduncle (ped.) are evident.

opaque. From 20 to 72 hours post-recovery, the carapace, natatory appendage exuvium and compound eyes were shed (Fig. 8B). The newly emerged adults exhibited stalk movement and slight cirral and valve movement. The naupliar eye and orange pigment were still visible and the five primordial valves evident (Fig. 8C). The orange pigment appears closely associated with the gut region.

Young adults exhibit little activity at first but between two to three days after metamorphosis a full extension of the cirri was noted. At this point the orange pigment has disappeared and the naupliar eye is indistinct. The young barnacles will readily feed on crushed *Artemia*.

DISCUSSION

Field results indicate that breeding of *O. mülleri* and cyprid settlement on crabs is restricted to summer and fall months. Although adults are often maintained in this laboratory at 10° C without apparent ill effects, release of larvae rarely occurs below 15° C and initial attempts to rear larvae at 15° C have failed. At this temperature the swimming motion of the larvae is slow and unsteady. Feeding is probably greatly reduced or impossible. The northern extension of *O. mülleri* appears to be limited by the temperature requirements for breeding and larvae development. The exact temperature requirements need to be established.

In most cases larval development of barnacles proceeds through six free-swimming naupliar stages and one cyprid stage prior to settlement and metamorphosis to the adult form (Newman, Zullo and Withers, 1969). Normally the stage I nauplius is nonfeeding and rapidly molts, while stages II–VI are actively feeding larvae filtering plankton. Several lecithotrophic larvae will develop without feeding (Batham, 1945a, 1945b; Anderson, 1965; Kaufman, 1965). *O. mülleri* nauplii swim dorsal side up directing fine particles toward the labrum by sweeping motions of their appendages. They appear to effectively filter dilute algal suspension and rapidly become fouled in dense cultures.

In laboratory cultures the development time of *O. mülleri* from newly-hatched egg to cyprid is 14–18 days at 24–29° C. This compares favorably with development times for local balanid species reared in the laboratory: *Balanus eburneus*, 7–12 days at 26° C (Costlow and Bookhout, 1957), *Balanus amphitrite denticulata*, 7–10 days at 26° C (Costlow and Bookhout, 1958) and *Balanus galeatus*, 9–14 days at 23–25° C (Molenock and Gomez, 1972). Extremes in development times of planktotrophic nauplii range from 22 hours for *Chthamalus stellatus stellatus* at an unspecified temperature (Daniel, 1958) to 57 days for *Lepas anatifera* at 20° C ± 3° C (Moyse, 1963). The extraordinary time cited by Daniel needs verification; however, monthly plankton samples in the North Atlantic support the findings of Moyse of a long larval life for *Lepas anatifera* (Bainbridge and Roskell, 1966). Although stage II *Lepas* larvae, ranging in length from 0.6 to 0.8 mm (Groom, 1894), are nearly equal in length to stage II *O. mülleri, Lepas* larvae are over 9 mm by stage VI (Moyse and Knight-Jones, 1967; Bainbridge and Roskell, 1966). *O. mülleri* has neither a long larval life nor exceptional growth increments.

Of the few barnacle nauplii described, *O. mülleri* most closely resembles the lepad larvae. The very long caudal spine and abdominal process are characteristic of both groups, as are relatively long setae and setules and long tufted frontolateral horns (Willemoes-Suhm, 1876). The difference in general appearance between these larvae and the short compact balanid larvae is striking, but is merely a result of proportional size differences in common structures.

O. mülleri larvae exhibit a curious underdevelopment of certain posterior features relative to other barnacle larvae. The mandibles remain small and show little change with successive molts, the exopodite, in particular, being greatly reduced. The mandibular gnathobase is also reduced, suggesting a possible modified feeding pattern. A rudimentary maxillule, a common feature of later stage nauplii (Bassindale, 1936; Bainbridge and Roskell, 1966), is absent as are the lateral paired abdominal spines previously mentioned. A clearly-defined posterior carapace border has been a consistant feature of stage IV larvae (Jones and Crisp, 1954; Barnes and Costlow, 1961) but is delayed until stage V in *O. mülleri*. The significance of this trend is unknown.

Settlement of *O. mülleri* appears to be highly specific for decapod gills. Reared cyprids do not settle on culture dish surfaces or excised crab gills but will settle on gills of live crabs. Metamorphosis has been observed in four individuals, but a means for continuous observation of settlement has not yet been devised. Metamorphosis of *Octolasmis* appears similar to *Lepas* (Newman *et al.,* 1969). The five primordial valves are distinct before the cypris carapace is shed but, unlike *Lepas,* the valves are separated by large interspaces. Further details on subsequent valve calcification in juvenile forms is now under investigation.

This paper is based on part of a dissertation to be presented to the faculty of the Marine Science Program, University of South Carolina, in partial fulfillment for the Doctor of Philosophy Degree. I wish to thank Dr. W. B. Vernberg, Dr. B. C. Coull, and Dr. P. A. Sandifer for reading and criticizing the manuscript and Mr. J. Takahashi for his initial help in maintaining the algae used for this project.

SUMMARY

1. The larval phase of *O. mülleri* consists of six naupliar stages and one cyprid stage. Descriptions and distinguishing characteristics are given for each stage.

2. Based on field observations and initial laboratory results, the breeding season of *O. mülleri* is probably restricted to summer months in temperate regions. Larvae appear unable to effectively feed at 15° C or below.

3. In small cultures, 93% of the larvae reached the cyprid stage using a mixed algae diet of *Tetraselmis suecia* and *Monochrysis* sp.

4. Larval development from newly-hatched nauplii to cyprid ranges from 14 to 18 days at room temperature (24–29° C) in laboratory culture.

5. Larvae most closely resemble described lepad larvae in general body form but have reduced mandibles and lack a series of abdominal spines reported for both balanid and lepad nauplii. The development of a posterior carapace border is delayed until stage V.

6. Reared cyprids were identical to "wild" specimens in morphology and size. Settlement of cyprid stages occurred only on live crabs. Some aspects of the external morphology of metamorphosis are described.

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MALATE DEHYDROGENASE ISOZYMES OF DIFFERENT STAGES OF CHESAPEAKE BAY JELLYFISH[1]

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In the Scyphozoa, pronounced morphological changes occur during strobilation which are undoubtedly preceded by changes in gene activity, metabolic pathways, and biosynthetic mechanisms: the long tentacles of the scyphistoma are retracted and a series of transverse constrictions are formed at the oral end; each transverse section develops into an ephyra and subsequently into an adult medusa. This morphological development of *Chrysaora quinquecirrha* has been thoroughly studied by Littleford (1939) and Calder (1972). Influences of environmental and chemical factors on strobilation of both *Aurelia aurita* and *C. quinquecirrha* have been investigated by many workers (Custance, 1964; Spangenberg, 1965, 1967, 1974; Loeb, 1970, 1973; Loeb and Gordon, 1975; Black and Webb, 1973; Olmon and Webb, 1974). Black (1972) reported increased levels of DNA in the strobilae of *A. aurita*.

Isozymes are probably important in regulating tissue function, and such studies may provide information on the molecular basis of cellular differentiation (Markert, 1975; Whitt, 1975). Investigations of the ontogeny of the most extensively studied enzyme, lactate dehydrogenase (EC 1.1.1.27), have been reviewed by Masters and Holmes (1972), and the evaluation of gene structure, function and regulation have been reviewed by Markert, Shaklee and Whitt (1975). The enzyme of interest, malate dehydrogenase (MDH, EC 1.1.1.37), has been studied in a few developing embryos of vertebrates, including the frog (MacBride and Guttman, 1973), fish (Nakano and Whiteley, 1965; Shaklee, Champion, and Whitt, 1974), and chicken (Greenfield and Boell, 1970).

Only a limited number of MDH isozyme studies have been undertaken on the development of vertebrates. These include the nematode, *Ascaris suum* (Zee and Zinkham, 1968), the marine snail, *Ilyanassa obsoleta* (Meizel and Markert, 1967), and the sea urchins, *Arbacia punctulata* (Moore and Villee, 1961, 1962, 1963a, 1963b; Billiar, Brungard, and Villee, 1964; Billiar, Zelewski, and Villee, 1966; Francesconi and Villee, 1968; Villee, 1968) and *Strongylocentrotus purpuratus* (Ozaki and Whiteley, 1970). In this study, the MDH isozyme patterns of different stages of the Chesapeake Bay jellyfish, *A. aurita, C. quinquecirrha,* and *Cyanea capillata,* were investigated as a model system for probing biochemical development of the simplest Animalia (Whittaker, 1969; Margulis, 1971).

MATERIALS AND METHODS

Medusae of *A. aurita* and *C. quinquecirrha* used in this study were collected from the York River, Virginia near the Virginia Institute of Marine Science in the summer of 1972. *C. capillata* medusae were collected during the previous winter season from the same location. Scyphistomae cultures of *C. capillata* and *A. aurita* were started from eggs collected from mature medusae (Calder, 1971). Planulae and planulocysts of *C. capillata* were obtained after placing fertilized eggs taken from medusae into culture. In the case of *C. quinquecirrha*, male and female medusae into culture. In the case of *C. quinquecirrha*, male and female medusae were put in the same container overnight and fertilized eggs were collected from the bottom of the container. Scyphistomae and cysts were obtained by maintaining planulae in filtered York River water (19–21‰ salinity) at 15° C for *C. capillata* and at room temperature (19–21° C) for *C. quinquecirrha* and *A. aurita*. The scyphistomae were fed nauplii of newly hatched *Artemia salina* once a week.

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[1] VIMS Contribution No. 737.

After washing, the organisms were ground with 3% (w/v) sucrose in 0.004 M Tris-0.038 M glycine buffer, pH 8.3 and the homogenate was centrifuged at 2000*g* for ten minutes. The crude extracts of different stages were applied directly to the tops of the gels without the aid of spacer gels. Polyacrylamide gel electrophoresis in a vertical cell (Büchler Instruments, USA), using 7.5% gel made with acrylamide (Eastman) and Tris-HCl-TEMED buffer system, pH 8.9, was performed (Davis, 1964). All gels were polymerized with ammonium persulfate rather than photopolymerization. Electrophoresis was performed at 5 mA per gel in a water-cooled cell (usually 2–3 hours) with bromophenol blue as the electrophoresis dye marker.

In order to determine whether the observed MDH isozyme differences were associated with the mitochondria or cytosol, mitochondria were prepared from medusae of *A. aurita* and *C. quinquecirrha*. The tentacles and oral arms of fresh medusae were mixed with 0.6 M sucrose-Tris-glycine buffer, pH 8.3 (1 : 1, w : v). After the homogenate was centrifuged at 2000*g* for five minutes, the supernatant was removed and the precipitate discarded. The 2000*g* supernatant was centrifuged in a Sorvall centrifuge at 7000*g* for thirty minutes, the supernatant discarded, and the precipitate (mitochondrial fraction) resuspended in fresh sucrose buffer and centrifuged again. After this washing process was repeated four times, the mitochondrial fraction was homogenized with 3% sucrose-Tris-glycine buffer, and the homogenate applied directly to the top of a 7.5% gel without the aid of a spacer gel. Electrophoresis was carried out as usual (Zubkoff and Lin, 1975).

RESULTS

Aurelia aurita

MDH isozyme patterns of *A. aurita* are shown in Figure 1. The scyphistomae possess four bands as described previously (Lin and Zubkoff, 1973). There is little difference in MDH isozyme patterns among planulae, scyhistomae, strobilae, and medusae. In these four developmental stages, the major isozyme is the 0.43 mitochondrial form. In *A. aurita,* the fast migrating bands (0.39 and 0.43) are the mitochondrial form and the slow moving bands (0.33 and 0.29) are the isozymes of the cytosol (Figure 2). Although this MDH isozyme pattern differs from that of most organisms in which the mitochondrial MDH moves more slowly toward the anode than the cytosol MDH, it is similar to that for tuna (Kitto and

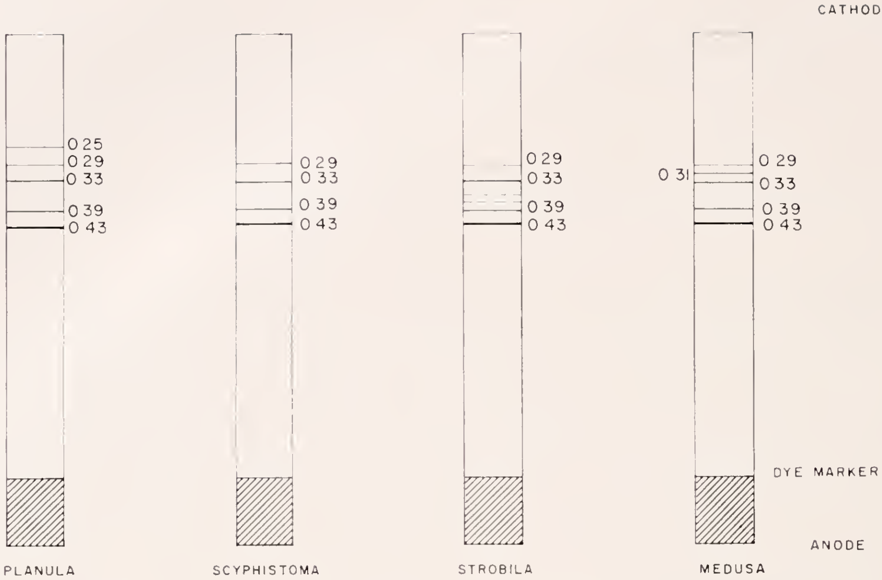

FIGURE 1. MDH isozyme patterns of different stages of *Aurelia aurita.*

Lewis, 1967), the marine snail, *Ilyanassa obsoleta* (Meizel and Markert, 1967) and the sea urchin, *Strongylocentrotus purpuratus* (Ozaki and Whiteley, 1970). Although no differences of the mitochondrial MDH (0.39 and 0.43) were observed among planulae, scyphistomae, strobilae, and medusae, the planulae do possess an additional slow moving band (0.25) which is lacking in the other polymorphs. The only difference among the strobilae is the presence of two trace bands which are not detected in any other stage. Of the thirty *Aurelia* medusae studied, two kinds of MDH isozyme patterns were observed: the isozyme pattern of the medusa in which an additional 0.31 band is present (Figure 1) represents twenty of the *A. aurita* medusa population studied, whereas the other ten medusae have the same isozyme pattern as the scyphistomae. Although ephyrae were not analyzed due to a lack of material, their MDH isozyme patterns would undoubtedly be the same as that of the scyphistomae and medusae.

Chrysaora quinquecirrha

The MDH isozyme pattern of developing *C. quinquecirrha* is shown in Figure 3. As previously described (Lin and Zubkoff, 1973), the scyphistomae have 7 bands: the major bands with strongest intensity of staining are the 0.53–0.54 doublet, and the 0.40 band. Although changes have occurred through the development of *C. quinquecirrha,* the only difference between scyphistomae and strobilae is that the strobilae have a slow-moving band (0.19). Whether this 0.19 band is due to dissociation of the 0.21 band, or is a band unique to the strobilae, is unknown at this time. During the ephyra stage, the intermediate migrating bands (0.31, 0.37, and 0.40) show a decrease in their activities. The 0.40 band which is intense in

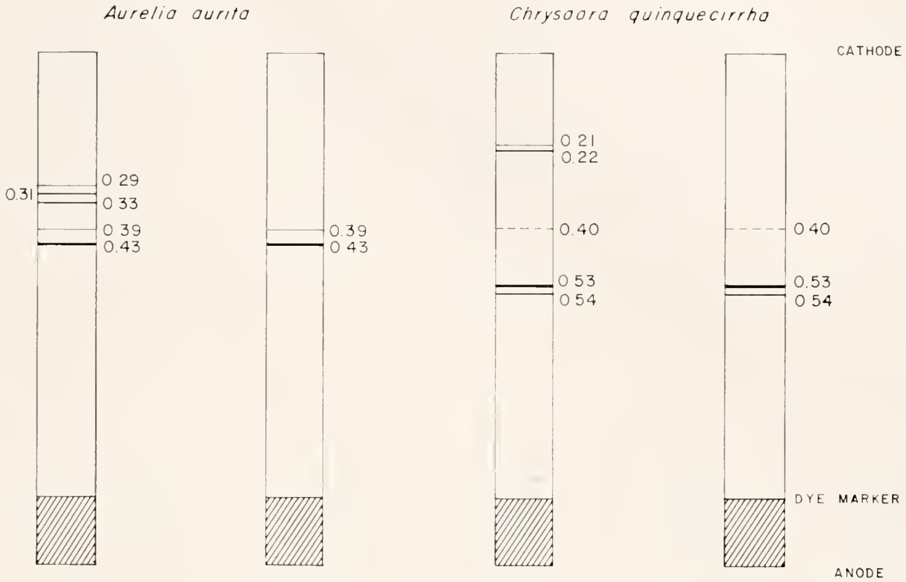

FIGURE 2. Mitochondrial MDH isozyme patterns of *Aurelia aurita* and *Chrysaora quinquecirrha* medusae. Left: whole tissue homogenate; Right: mitochondrial fraction.

the scyphistomae can be observed only as a trace band in the ephyrae; the 0.33 band of the scyphistomae is not observed in the ephyrae. In the medusae, there is a further reduction in the activities of the intermediate migrating bands clearly observed in the scyphistomae and strobilae: only the 0.40 band can be observed, whereas the other three bands (0.31, 0.33, and 0.37) are absent. In these four stages, scyphistomae, strobilae, ephyrae, and medusae, the major bands are at 0.53 and 0.54. In medusae, 0.40, 0.53, and 0.54 bands are the mitochondrial MDH and 0.21 and 0.22 bands are MDH of the cytosol (Figure 2). In addition to the loss of the intermediate migrating bands (in progressing from scyphistomae to the medusae), there is also an increase in the activities of the cytosol MDH (0.21 and 0.22 bands) in the medusae. Thus, there is a shift from many MDH isozymes in the scyphistomae to fewer isozymes in the medusae. In podocysts, the slow migrating doublet (0.21–0.22) associated with the cytosol predominates; the other bands associated with the scyphistomae and medusae are not detected.

At times, both red-pigmented and white-pigmented *Chrysaora* medusae occur. Although we suspect that this pigmentation is due to either a genetic difference and/or the influence of environmental factors such as food and light, a single MDH isozyme pattern is observed in both the white and red *C. quinquecirrha* medusae populations.

Cyanea capillata

Lin and Zubkoff (1973) observed that the scyphistomae of *C. capillata* have a single fast moving band (0.50) of great staining intensity and a slow moving

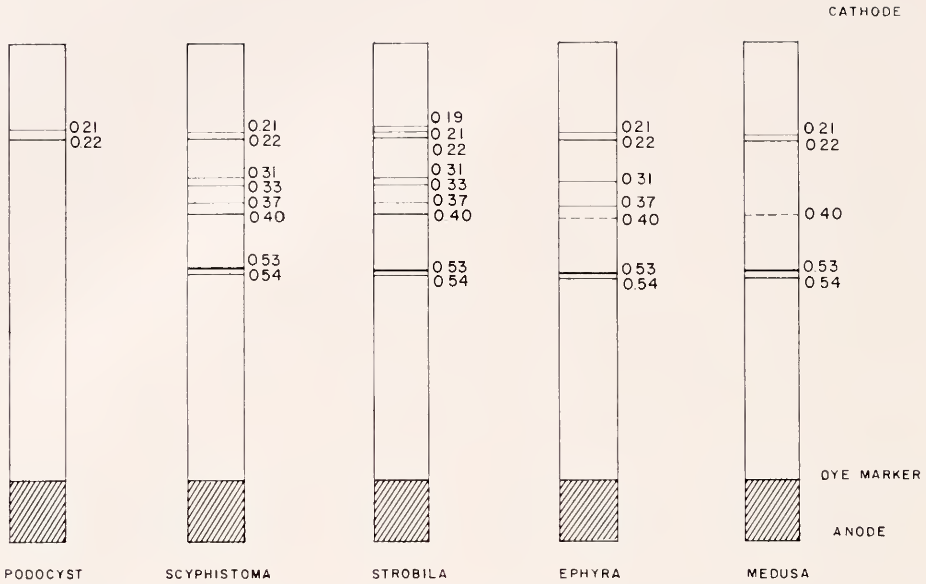
CATHODE
0.21
0.22
0.19
0.31
0.33
0.37
0.40
0.53
0.54
DYE MARKER
ANODE
PODOCYST
SCYPHISTOMA
STROBILA
EPHYRA
MEDUSA

FIGURE 3. MDH isozyme patterns of different stages of *Chrysaora quinquecirrha.*

triplet (0.25). The medusae of *C. capillata* have the same MDH isozyme pattern as the scyphistomae, but there is an increased activity associated with the slow moving bands (inferred to be cytosol in anology with that of *A. aurita* and *C. quinque-*

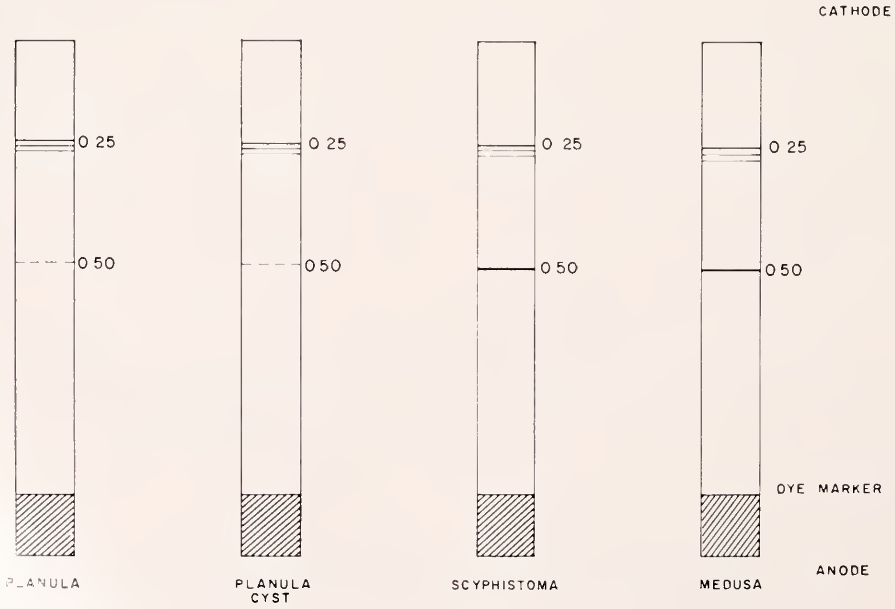
CATHODE
0.25
0.50
DYE MARKER
ANODE
PLANULA
PLANULA CYST
SCYPHISTOMA
MEDUSA

FIGURE 4. MDH isozymes patterns of different stages of *Cyanea capillata.*

TABLE I

Summary of MDH isozyme patterns of different stages of Chesapeake Bay jellyfishes. Parenthesis indicate the number of homogenates prepared; brackets, the number of organisms in each homogenate; face brackets, μg of protein in homogenate. The a indicates polymorph not observed; b, not available in sufficient quantity; c, trace bands appear in some preparations; d, inferred (see Discussion).

	Planula	Cyst	Scyphistoma	Strobila	Ephyra	Medusa
Aurelia aurita	(7) [20–40] {40–60}	a	(30) [7–9] {20–30}	(4) [6–9] {20–30}	b	(30) [<1] {100–200}
0.25	+					
0.29	+		+	+		+
0.31						+++
0.33	+++		+++	+++		+
0.36				c		
0.38				c		
0.39	++		++	++		++
0.43	++++		++++	++++		++++
Chrysaora quinquecirrha	b	(18) [40–60] {120–340}	(41) [7–9] {20–30}	(16) [6–9] {20–30}	(11) [20–40] {10–25}	(30) [<1] {100–200}
0.19	++d	++	+	+		
0.21	++d	++	+	++	+	++
0.22	++d	++	++	++	++	++
0.31			++	++	+	
0.33			++	++		
0.37			+	+	+	
0.40			+++	+++	+	c
0.53	+d		++++	++++	+++	++++
0.54	+d		+++	+++	++	+++
Cyanea capillata	(14) [30–60] {40–60}	(7) [40–60] {120–200}	(23) [15–20] {20–30}	b	b	(12) [<1] {100–200}
0.25a	++++	++++	+			++
0.25b	+++	++	+			++
0.25c	++	++	+			++
0.50	c	c	+++			++++

cirrha). Although the planulae and planulocysts of *C. capillata* have the same isozyme patterns as the scyphistomae, there is also a shift in the intensity of the fast and slow migrating bands: the slow migrating bands are the dominant form and only a trace amount of the fast moving band exists in the planulae and planulocysts. This transition of MDH isozyme dominance from planulocysts to scyphistomae is clearly demonstrated in the newly emerged scyphistomae: the 0.50 band, the band that is dominant in aged scyphistomae, is as intense as the 0.25 isozyme triplet. Within the medusae population analyzed, only one isozyme pattern was observed.

In contrast to MDH isozyme patterns which are summarized in Table I, the glucose-6-phosphate dehydrogenase (G6PD, EC 1.1.1.49) of *A. aurita* has only one band (0.20) (Zubkoff and Lin, 1975). The G6PD of *C. quinquecirrha* and *C. capillata* also has a single band (0.20) and no change in the G6PD isozyme patterns is observed during the developmental stages of these three species.

Discussion

Changes in isozyme patterns indicate gene expression and may reflect genetic or epigenetic events taking place within the organism (Whitt, 1975). In this study, MDH isozyme changes during the process of strobilation are reported. In the scyphistomae of *A. aurita*, two intermediate migrating bands are found. In the scyphistomae of *C. quinquecirrha*, an additional trace band (0.19) is observed. Although the interpretation that the appearance of the new MDH isozyme is probably due to derepression of the gene is favored, it is possible that either the slowing down of the rate of degradation of that isozyme, the presence of inhibitors, or an epigenetic event may change the isozyme patterns during development. Dissociation or association of an isozyme subunit caused by the binding of metal ions or other compounds which change the electrophoretic mobility of that isozyme is not ruled out.

The MDH isozyme patterns of developing *C. capillata* reveal changes. In the four developmental stages studied, all have two groups of bands (0.25, 0.50). However, there is a shift from the dominant slow moving triplet of MDH of the planulae and planulocysts to the multiple forms of MDH of the scyphistomae and medusae. Whether this transition is due to the change in isozyme activities or the amount of isozyme, is still unknown. It is apparent that all the genes which are responsible for the synthesis of these isozymes are activated in these developmental stages.

The MDH isozyme pattern changes in different stages of *C. quinquecirrha* reveal that the mitochondrial MDH dominate in the scyphistomae and medusae. But the presence or absence and the relative activities of certain isozymes contribute to its complexity. Only minor differences between scyphistomae and strobilae are observed with pronounced MDH isozyme patterns first appearing in the ephyrae. The disappearance of the 0.33 band and the decreasing activities of other intermediate migrating bands are the major evidence of the change of the MDH isozyme pattern. After the ephyrae mature to medusae, the difference is more pronounced: there is only one intermediate migrating band (0.40) in the medusa, the rest of these intermediate migrating bands (0.31, 0.33, and 0.37) have disappeared either by degradation, inhibition, or lack of synthesis. On the other hand, there is an increase in the MDH of the cytosol in the medusae. Unfortunately, because of a lack of planulae of *C. quinquecirrha*, MDH isozyme analysis could not be undertaken. The planula stage which serves as a transition stage between eggs and scyphistomae is very short. However, from the data obtained from *C. quinquecirrha,* and the evidence from *C. capillata,* one can infer that, in planulae, the dominant bands would be the 0.21–0.22 doublet of the cytosol and that the activities of the other isozymes are increasing.

The increased mitochondrial MDH in the scyphistomae and medusae of *C. quinquecirrha* is certainly associated with energy production. When the animal is dormant, as in the cyst, it requires very little energy for maintaining its metabolic activities. When the planulocyst transforms into a scyphistoma, a greater energy requirement exists for maintaining its normal activities, and probably an even greater energy requirement is necessary in the strobilae and medusae. Thus, the genes for mitochondrial MDH are activated and responsible for the production of more energy and biosynthetic precursors.

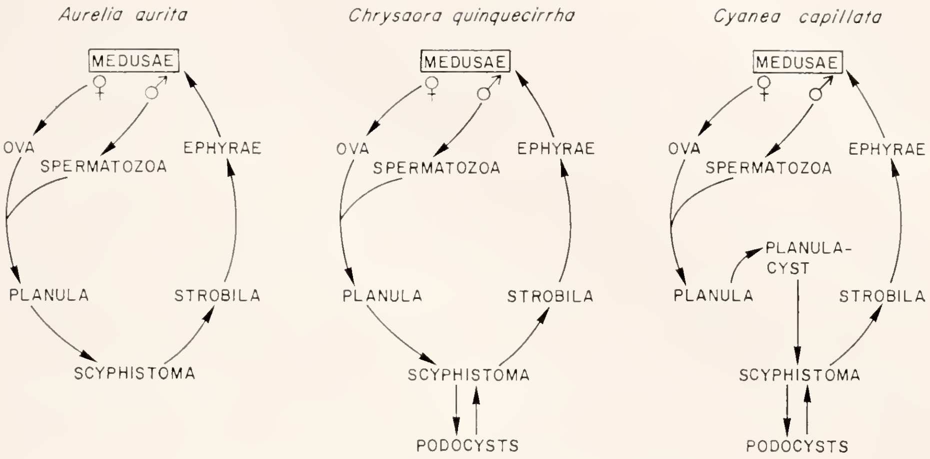

FIGURE 5. Life cycles of Chesapeake Bay jellyfish.

In *A. aurita*, the change of the MDH isozyme pattern is restricted to the cytosol MDH. Because the genes which are responsible for the synthesis of mitochondrial MDH are always in the same activated states, the explanation that certain genes for energy production are activated is not applicable. It should be noted that differences in the life cycles of these three Chesapeake Bay jellyfish exist (Figure 5). *C. capillata* planulae develop into planulocysts, which undergo further development into scyphistomae. The *C. quinquecirrha* planulae develop directly into scyphistomae, without formation of the planulocysts, and the scyphistomae normally form numerous podocysts. Furthermore, the scyphistomae may undergo transformation to form podocysts under adverse conditions, and may revert back to scyphistomae when the adverse conditions are removed. In contrast, cyst formation in the life cycle of *A. aurita* from Chesapeake Bay has not been observed. This may account for differences in the MDH isozyme patterns changes in *A. aurita* when compared to the other two scyphozoans.

The electrophoretic pattern of glucose-6-phosphate dehydrogenase is unlike that of other enzymes of scyphozoan jellyfish (Lin and Zubkoff, 1973; Zubkoff and Lin, 1975). MDH, superoxide dismutase (SOD, EC 1.15.1.1 = terazolium oxidase), and esterase of Chesapeake Bay jellyfish all have isozyme patterns whereas G6PD appears as a single band in the polyacrylamide gel enzyme assay. Although Blanquet (1972) indicated that scyphistomae of *C. quinquecirrha* have an increased G6PD activity during cold acclimation, there is no electrophoretic change in the G6PD pattern during the development of these three species. Blanquet (1972) also suggested that the increased G6PD activity makes more NADPH available for synthesis of fatty acids to be stored in the cysts. Since MDH of jellyfish can use either NADP or NAD as a co-factor (Lin and Zuboff, 1973), it may also serve as a potential NADPH supplier for this purpose.

The Chesapeake Bay jellyfish medusae appear at different seasons: *A. aurita* and *C. quinquecirrha* appear in the summer and *C. capillata* in the winter. The MDH isozyme pattern changes during development are very different between these two summer jellyfish. In *A. aurita,* the changes are small and confined to the cytosol MDH. In *C. quinquecirrha,* changes of the intermediate migrating MDH isozymes (0.31, 0.33, 0.37, 0.40) occur. In *C. capillata,* the presumed MDH isozymes (0.25) of the cytosol are more prominent in the planulae and planulocysts whereas the presumed mitochondrial MDH (0.50) is most prominent in the scyphistomae and medusae.

Different MDH and SOD isozyme patterns are associated with scyphistomae from distant geographical locations which have significantly different seasonal patterns (Zubkoff and Lin, 1975). However, it is doubtful that temperature directly influences the change of these MDH isozyme patterns during development. In order to study the short term temperature effect on the MDH isozyme patterns of *C. quinquecirrha* and *A. aurita,* the scyphistomae of these two species cultured under different temperatures were compared. The results showed that scyphistomae maintained at both a lower temperature (12–15° C) and at room temperature (18–21° C) for one year have the same isozyme patterns. However, the substrate affinity and the free energy of activation of MDH in cold and warm cultured scyphistomae are different (Lin and Zubkoff, in preparation).

Numerous questions remain unresolved with respect to the development of jellyfish. In *C. quinquecirrha,* can scyphistomae derived from planulae be distinguished from scyphistomae derived from podocysts? Can scyphistomae which have undergone strobilation and reverted back to scyphistomae be differentiated from scyphistomae which have never strobilated? Since there is no structural difference between these scyphistomae, physiological, biochemical and genetic approaches must be employed.

Parts of this study were carried out under Public Law 89–720, The Jellyfish Act, in cooperation with the National Marine Fisheries Service of the U. S. Department of Commerce and the Commonwealth of Virginia. We thank Dr. R. E. Black for reviewing the manuscript and Mr. Michael A. Gorey for maintaining the cultures.

SUMMARY

1. In *Aurelia aurita,* there is no change in the mitochondrial MDH isozyme pattern in different stages, although a relatively small change in the cytoplasmic MDH isozyme pattern can occur.

2. In *Chrysaora quinquecirrha,* three MDH isozyme patterns are observed: a dominant cytosol pattern in the cysts, a complex pattern in the scyphistomae and strobilae, and a simpler cytosol and mitochondrial pattern in the ephyrae and medusae.

3. In *Cyanea capillata,* two MDH isozyme patterns occur in the different stages: the planula and planulocyst pattern and the scyphistoma and medusa pattern.

4. Temperature induced differences were not observed in MDH isozyme patterns of the scyphistomae of *A. aurita* and *C. quinquecirrha* when cultured under different temperatures.

5. No differences in the MDH isozyme patterns of the red and white medusae of *C. quinquecirrha* were detected.

6. Changes in G6PD enzyme electrophoretic mobility during the development of *A. aurita, C. capillata* and *C. quinquecirrha* were not observed.

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TRANSPORTATION AND MAINTENANCE OF ADULT SQUID (*DORYTEUTHIS BLEEKERI*) FOR PHYSIOLOGICAL STUDIES

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Almost all the important problems in nerve physiology have been successfully approached through the study of animals with particularly large nerve cells or fibers that are amenable to experimentation. Two nerve fibers in the nervous system of the squid are the largest; they are often called giant nerve fibers. Their diameters are between 400 and 800 μ. A single nerve fiber from the live squid is easily obtained. The important characteristics of a single nerve fiber have been found or ascertained in the squid giant axons (Rosenberg, 1973). Only the squid giant axons have clarified basic processes associated with nerve excitation and with nervous conduction (Tasaki, 1968; Hodgkin, 1964).

Squid experiments are usually limited both regionally to some marine laboratories and seasonally by availability of natural squid stocks (Arnold, Summers, and Gilbert, 1974) (in our case in Japan, it is usually from October through December). These limitations can be overcome if the squid could be transported from several fishing sites with different squid seasons to a well-equipped laboratory, and if the squid could be maintained in an aquarium in the laboratory. Transportation and maintenance of the squid is one of the basic requirements for any study of squid axons in any laboratory located far from the sea (Spotte, 1973).

Here, successful experiments on the transportation and maintenance along these directions are reported.

MATERIALS AND METHODS

Source of squid

Squid used in the maintenance study and subsequent physiological experiments were all *Doryteuthis bleekeri,* purchased from commercial fishermen operating near Jogashima Island, Kanagawa prefecture. They were caught in a seasonal fishery (October through December) using Japanese squid jiggers of the so-called "single line" technique. Individual jiggers are elongated, colored plastic lures about 9.0 cm long, with two circles of barbless hooks around the lower end. Ten to fifteen squid were purchased weekly in 1973 and 1974, resulting in a cumulative total of 292 experimental animals. Ambient conditions on the fishing grounds were measured on November, 1973; with the following results: depth of squid, 100 m or less; water temperature, 13° C; dissolved oxygen, 4.11 ml/l.

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Transportation

Squid were transported by truck using one of two kinds of containers. Initially (October and November, 1973), 40 cm diameter × 1m bags made of vinyl chloride were used. The bag was half-filled with filtered sea water, and two or three squid were gently put into it. The bag was then inflated with high pressure oxygen gas and put into a corrugated cardboard box. This is similar to the method used to transport live carp or goldfish, and is especially suitable for small numbers of squid.

After November 1973, an alternative mass transportation method was adopted, which was preferable since enough sea water to fill our aquarium could be obtained at the same time. Filtered sea water was taken from the Kanagawa Prefectural Fisheries Experimental Station at Jogashima Island before the squid purchase. A tank composed of a 1m × 1m × 1m skeleton box and dark green vinyl cloth carried both sea water and ten to fifteen squid. It was thought that the soft configuration might mitigate damage to the squid as a result of collisons with the tank walls. In transport, the tank was filled to the top to prevent splashing and any resultant stimulation of the squid. Oxygen gas was continuously bubbled into the tank from a high pressure source. The temperature of the sea water in the tank was 17 to 19° C and was not controlled. After a three to five hour trip, the squid were quickly transferred into a prepared aquarium. The trip was made once a week on an average, and the total number of trips in 1974 was 12. No deaths during the transport of the squid were observed.

Maintenance system

The maintenance system was located in a small wooden house approximately 3.2 m long by 3.8 m wide, especially built for this study. The windows were covered with black papers so that no direct sunlight could shine into the room, and it was kept in almost total darkness at all times when squid were present, which made the environment resemble the squid's natural habitat about 100 m from the sea surface.

The aquarium system consisted of a circular tank with lid, three filtering stages, an activated carbon filter, temperature control, air bubbler, and circulation pump (see Figure 1a). The outlet of the filtered, circulating sea water was directed so that it would flow peripherally around the circular aquarium (see Figure 1b). The flow velocity of circulating sea water was 5 m/min. This flow pattern may decrease squid collisions with the aquarium walls, since they appear to align themselves with the direction of flow. In 1975, a net pattern was drawn with black-colored vinyl paint on both inner and outer aquarium walls. This, in addition to the circulation of the sea water, completely prevented the squid from even touching the walls. Both outer and inner walls of the aquarium were made from polyethylene. The outer diameter was 1.5 m and the inner diameter was 0.5 m; the depth was 1.0 m. The lid satisfactorily prevented squid from getting out of the aquarium.

Each filtering stage was composed of layers of small stone, glass wool, activated charcoal, and several sizes of sand (Ooiso sands), as shown in Figure 1a. The area of filter 10A was 50 cm × 100 cm; depth was 45 cm. It was composed of two sizes of sand: for one the diameter was 0.5 mm, and for the other 1 mm, on an average. The thickness of each layer was 15 cm for coarser size of sand and 25 cm for finer one. The coarser size of sand was under the finer.

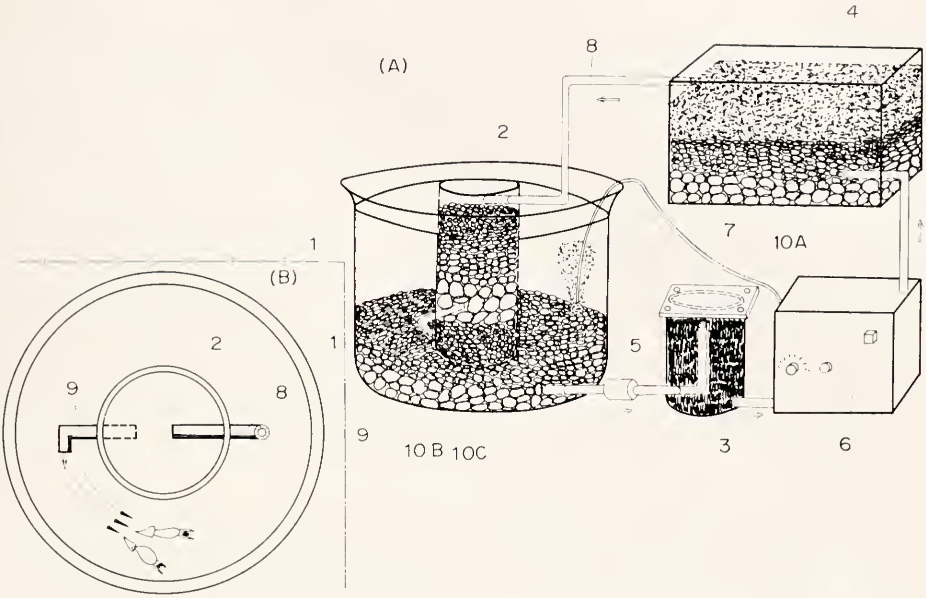

FIGURE 1. (a.) Configuration of the aquarium system. 1, shows the aquarium; 2, central cylinder which prevents squid movement in a radial direction and also serves as a container for filtering stage 10C; 3, activated charcoal filter; 4, filtering stage 10A; 5, dirt trap; 6, control box for circulation pump and temperature control unit; 7, air bubbler; 8, overflow from filter 10C; 9, seawater outlet; 10A, 10B, and 10C, three filtering stages. (b.) Circulation pattern of sea water in the aquarium, plan view.

Filter 10B was composed of layers of small stones (diameter of 5 mm, and thickness of 5 cm); glass wool (thickness of 5 mm when not wet); and sand of grain diameter 2 mm (thickness, 25 cm). The area of filter 10B was 1.5 m^2.

Filter 10C was composed of layers of small stones (diameter of 5 mm, and thickness, 10 cm); glass wool (thickness, 5 mm); activated charcoal (cylindrical pieces with diameters of 2.5 mm, and length of 4 mm; thickness of the layer, 20 cm); and sand (diameter of 2 mm on an average, and thickness, 40 cm). A layer of commercially available Zeolite (diameter of 4 mm and thickness, 5 cm) was added in 1975 to the above consituents of filter 10° C to moderate pH change.

The temperature control unit can regulate the sea water temperature in the aquarium at specific temperatures in the range 10° C and 25° C with an accuracy of ± 0.2° C. The circulation pump circulated the sea water at the rate of 200 l/min for the system (Spotte, 1973; Mariscal, 1974).

RESULTS

The heavy solid line in Figure 2 shows a typical percentage of survival in relation to number of maintenance days, obtained from a total of twenty squid in 1974. In 1974, undisturbed observations on the maintained squid were made on two sets of seven days, the initial week and the last week of the squid season. For both trials, one squid out of the ten maintained was dead on the seventh day. The remaining squid were used for our subsequent physiological experiments on the methods of observation. Therefore, an average survival percentage could not be obtained accurately, although the total number of the squid we used in 1974 was 192. For the first trial in 1975, the observation period was increased to three weeks without disturbance. For this trial, slices of raw tuna of approximately 300 g in total weight were given to 12 squid for three days as food. No natural death occurred during the three weeks except that one squid devoured another. In all other cases, the observation period was limited to one week because in the subsequent physiological experiments with squid giant axons, a consistent supply of eight to twelve live squid per week was found to be sufficient. Furthermore, many difficult problems associated with longer periods of squid maintenance were anticipated, such as regulation of sea water pH and salinity. For the present purpose of physiological studies, survival rates of 90% or better were satisfactory. Accordingly, the holding capacity of the aquarium system was designed originally to maintain ten to fifteen squid, and weekly purchases were anticipated.

Loss of squid during transportation did not occur, even though the fishing method and truck equipment were relatively crude operations. These conditions excited and/or tired the squid, because they appeared less vigorous and were alarmed by slight changes in their surroundings. For example, they would try to escape from 3- to 4 cm goldfish placed in the aquarium for food within the first day after arrival in the laboratory; some even inked under these circumstances. Squid would take goldfish on the second day, although we only repeated attempts to feed them on two occasions. At about the same time, the squid appeared much more accustomed to the aquarium environment and had recovered from their lack of vigor. Mating and spawning in the aquarium were often observed.

The aquarium system was usually preset to maintain a sea water temperature of 15° C, and the air bubbler was regulated to hold the dissolved oxygen level at about 4.1 ml/l. Temperature effects the survival mainly through associated changes in the dissolved oxygen concentration. On one occasion, almost all of the squid in the aquarium were moribund three hours after the air bubbler stopped.

Filtering capacity was the most important factor in the successful maintenance of squid. A single filter stage was used before the 1974 season (filter 10A, as shown in Figure 1). Survival of squid under these conditions is shown by the dashed line in Figure 2; half of the squid died in about one day. At the beginning of the squid season in 1974, filter 10B was added (see Figure 1), with no appreciable difference in survival rate over the previous experience. Soon thereafter, filter 10C was added, and a considerable improvement in survival was observed. That is, no natural death occurred within the first four days after stocking the aquarium and only 8% died within the first week from the 192 squid total. A typical survival pattern is shown as the heavy solid line in Figure 2. Change in survival is considered to be a discontinuous function of filtering capacity, as suggested by these results.

The conditions provided by the aquarium made collisions with the tanks walls infrequent. The fact that the squid oriented to the imposed circular flow was readily confirmed by reversing the outlet (number 9 in Figure 1) and observing

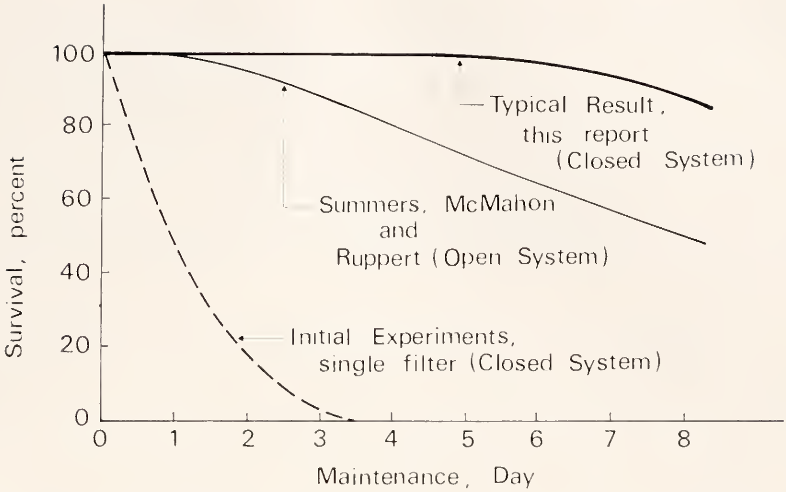

FIGURE 2. Squid survival over an eight day period. The heavy solid line shows experimental results for the system shown in Figure 1. The dashed line corresponds to the squid survival with the initial system before the addition of filtering stages 10B and 10C. The fine solid line shows results reported by Summers, McMahon and Ruppert (1974) using an open system and a bumper inside the tank walls.

that the squid quickly changed their direction of swimming. It was concluded in the initial stages of work in 1973 that avoidance of collision would not be a crucial factor for survival of squid, and efforts were concentrated on improving the filter capacity.

There was no appreciable difference in survival when the sea water temperature was varied over the range from 13° C to 20° C if enough oxygen was dissolved in the sea water, as was observed by Summers and McMahon (1970, 1974). However, there seemed to be a general tendency for spawning to occur at temperatures lower than 15° C.

DISCUSSION

Systematic work on the maintenance of squid has been extensively reported by Summers and McMahon (1974) and Summers, McMahon and Ruppert (1974) on the northwest Atlantic squid, *Loligo pealei*. They concluded that observed improvements in survivorship of squid were due to modifications in the capture techniques, sorting of squid, and the introduction of a bumper system. Their median survival time (when survival becomes 50%) was eight days; their data are shown in Figure 2. They used an open system, where running sea water fully replaced the tank volume 1.33 to 2 times per hour. Their bumper system was composed of polyethylene tubing suspended inside the walls of rectangular tanks (Summers, McMahon and Ruppert, 1974), which absorbed the impact of squid collision with the barrier. Their method of capturing squid differed from the one described in this report.

Additional filtering stages were effective in maintaining a viable sea water quality and promoting longer squid survival. The degree of the improvement is difficult to determine quantitatively. It can be concluded from the experimental results that there is a critical amount of filtration necessary for the maintenance of the squid; in other words, a threshold level exists. This threshold correlates to the squid's anatomical structure. The gills are directly exposed to ambient sea water and may become fouled by particulate matter in the water through normal activity or respiration. Fine dirt of glass wool in the gills of moribund individuals was often observed, using an optical microscope. The experimental results also correlate with the fact that the squid are found in clear sea water at depths up to nearly 100 m.

It is noted here that success in the maintenance of squid represents a single parameter solution. For more complete success, many other factors, such as pH control, salinity regulation, feeding, and associated sea water quality parameters, will likely be involved. From the experimental result in 1975, despite only one trial, for longer periods of observation than a week, it can be safely concluded that sufficient feeding is another important factor in maintaining squid for longer periods. After giving raw tuna to the squid for food, the sea water in the aquarium should be allowed to overflow to eliminate oil sheets on the surface of the sea water. Detailed studies for longer periods of maintenance are left for future work.

The author would like to express his cordial thanks to Dr. J. Shimada for his collaboration at the initial stage of this study and his sincere encouragements throughout it; to Dr. Konrad Lorenz for his sincere discussions and especially for his valuable suggestion that the squid might require plenty of food in view of their rapid growth to adult size in only one year; and to Professor W. C. Summers for his valuable discussions and his help in revising this manuscript. The author also thanks Mr. H. Shimizu for his continuous encouragement of this work.

SUMMARY

Transportation and maintenance of adult squid (*Doryteuthis bleekeri*) was investigated successfully. Squid were transported on a three to five hour trip and could then be maintained in circulating sea water in a closed system aquarium for up to three weeks. The most crucial factor for maintenance of squid was filtration of the sea water. The survival percentage of squid was 90–100% over the first week.

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EFFECTS OF SALINITY AND TEMPERATURE ON THE DEVELOPMENT OF EGGS IN THE TUBE BUILDING AMPHIPOD *COROPHIUM TRIAENONYX* STEBBING

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The amphipod *Corophium triaenonyx* is a fouling organism living in parchment tubes on timber and concrete jetties. It is a hardy form, tolerating wide ranges of salinity and temperature under laboratory conditions (Shyamasundari, 1973). It occurs abundantly throughout the year with peak numbers between November and March (Shyamasundari, 1972).

It is generally agreed that salinity and temperature play a major role in the development of embryos. The rate of embryonic development has been reported in several cases to be affected by salinity and the variation in salinity may modify the time and length of breeding season. Temperature fluctuations may enlarge or narrow the salinity range of the species and the salinity in turn may influence the temperature tolerance (Kinne, 1952; 1953a; 1956a; 1957; 1958a; and 1959). There have been quite a number of studies on reproduction and development in amphipods but contributions towards the effects of salinity and temperature or the combined effects of these parameters on development have been documented by a few papers. Most of the literature is based on field observations. Clemens (1950) studied the effect of salinity and temperature on the incubation period (time between fertilization and hatching) in *Gammarus fasciatus*. Bovie (1951) dealt with the pre-embryonic, post-embryonic and adult stages of *Hyalella azteca*. Kinne (1953a; 1959; 1960; 1961; and 1964) furnished information on the effects of salinity and temperature and their combined effects on the development of embryos, number of eggs and upper limiting temperature for embryonic development in *Gammarus duebeni*. Hynes (1954) showed the differences in the capacity of egg laying between freshwater and brackish-water forms of *Gammarus duebeni*. McLusky (1967; 1968) has given some details about the effects of salinity on survival, molting and growth activities of *Corophium volutator*. Vlasblom and Bolier (1971) studied the tolerance of embryos of *Marinogammarus marinus* and *Orchestia gammarella* to low salinities. The duration of embryonic development at various temperatures in *Gammarus duebeni, Gammarus lawrencianus, Gammarus obtusatus, Gammarus oceanicus* and *Gammarus setosus* was studied by Steele and Steele (1973). Dorgelo (1974) discussed the effect of salinity and temperature combinations on *Chaetogammarus marinus, Gammarus tigrinus* and *Gammarus forsarum* from marine, freshwater and brackish-water, respectively. Shyamasundari and Hanumantha Rao (1974) furnished some details about the effects of temperature on the embryonic and post-embryonic stages of *Corophium triaenonyx*.

An experimental study has been undertaken in order to ascertain more precisely the effects of salinity and the combined effects of salinity and temperature on the development of eggs in *C. triaenonyx*.

MATERIALS AND METHODS

Specimens of *C. triaenonyx*, after collection from jetties in the Visakhapatnam Harbor, were transferred to stock tanks. Berried females were separated and maintained in large tanks to obtain fertilized eggs. All the eggs in a single brood were in the same stage of development. Removal of eggs from the brood pouch with a sterile needle was done according to the method followed by Shyamasundari and Hanumantha Rao (1974). Usually eggs at 64 cell stage were used for all experiments. Fifty eggs were used in each experiment.

To each of the fingerbowls was added 50 cc of sea water of known salinity. A series of salinities covering a range from 7.5‰ to 37.5‰ at 2.5‰ intervals was prepared. Different concentrations were prepared as described by Shyamasundari

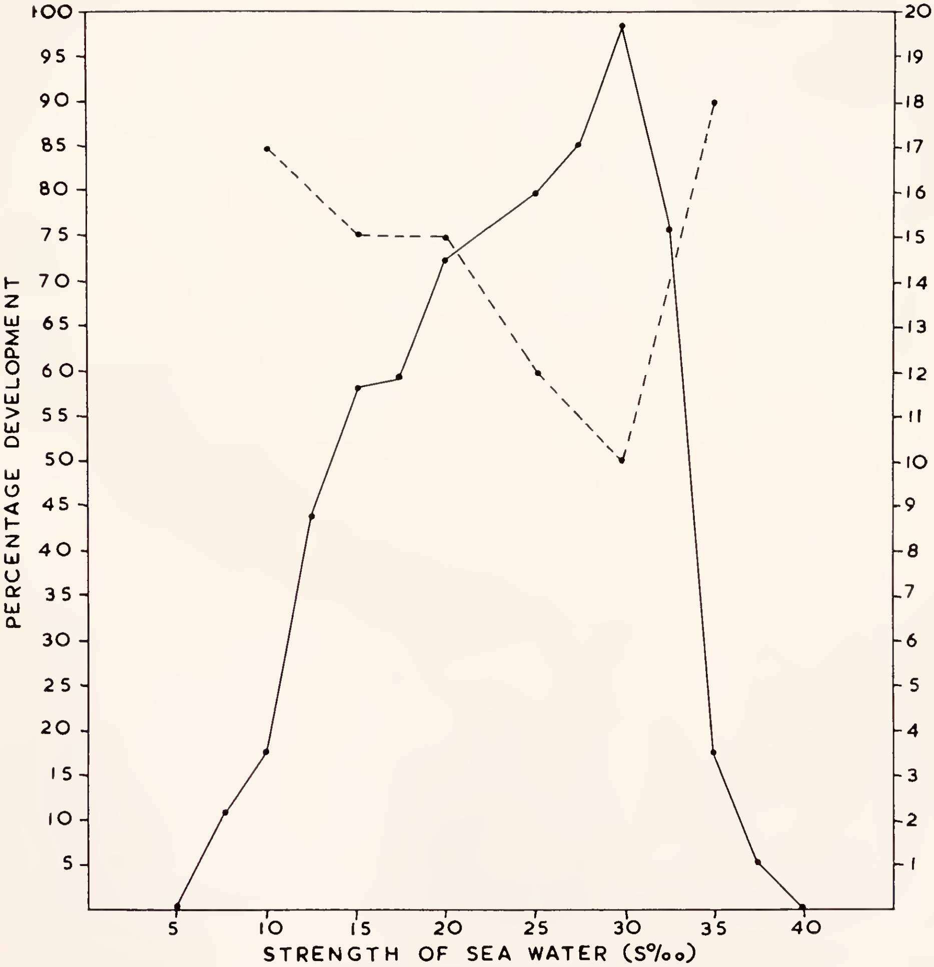

FIGURE 1. Effect of salinity on the percentage development and incubation period of eggs of *Corophium triaenonyx* Stebbing. The temperature was held constant at 28° C (±0.1° C).

(1973). Each experiment was repeated three times. All cultures were maintained at a constant temperature of 28° C (± 0.1° C). The dishes were examined daily and the degenerating eggs were removed. Water was changed every day.

To test the combined effect of salinity and temperature, six salinities (10‰, 15‰, 20‰, 25‰, 30‰, and 35‰) and five temperatures (9° C, 15° C, 22° C, 28° C, and 35° C) were chosen. Duplicate cultures were established at each of the six salinities and five temperatures tested, giving a total of 30 combinations and 60 cultures in each experiment. For all experiments controls were maintained. Normally, development in amphipods is completed in ten days time at salinity 30‰ and temperature 28° C. For convenience the entire development was divided into three stages: Stage I, advanced gastrula stage; Stage II, formation of rudimentary appendages; and Stage III, formation of eye.

RESULTS

Corophium triaenonyx eggs developed into young ones throughout a wide range of salinities from 7.5‰ to 37.5‰ at 28° C (± 0.1° C) maintained in the laboratory. At a higher concentration of 40‰ and a lower concentration of 5‰ development ceased. At 37.5‰ only 5.3% of the embryos developed into normal young ones and at 35‰, 17.5% developed normally. At 7.5‰ and 10‰ only 10.5% and 17.5%, respectively, of the embryos developed normally. Approximately 44.5% of the embryos developed into young ones at 12.5‰ but the salinity range for a satisfactory development of at least 58.5% was between 15‰ and 17.5‰. Similarly more than 70% of the embryos developed into young ones between 20‰ and 32.5‰, but the salinity range for a very high percentage (80%–95%) was between 27.5‰ and 30‰. With temperature constant at 28° C (± 0.1° C), the optimum salinity range for embryonic development is 27.5‰ to 30‰. The results are presented graphically in Figure 1.

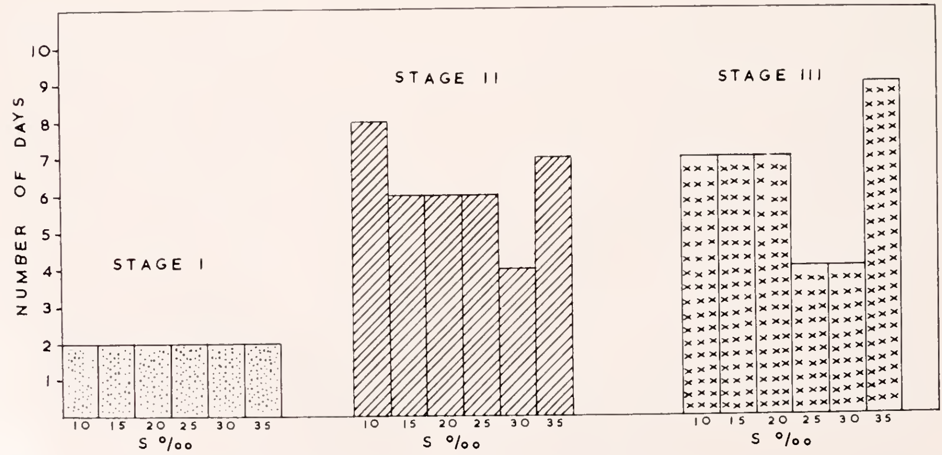

FIGURE 2. Effect of salinity on various stages of development of eggs of *Corophium triaenonyx* Stebbing. The temperature was held constant at 28° C (±0.1° C).

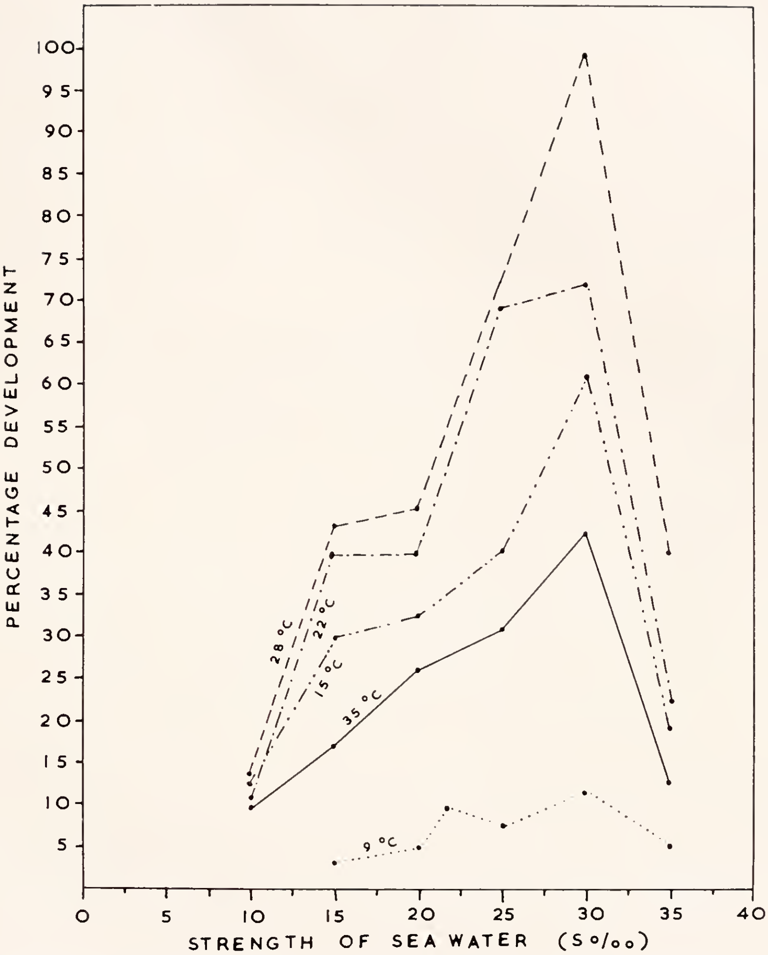

FIGURE 3. Combined effect of salinity and temperature on the percentage development of eggs of *Corophium triaenonyx* Stebbing.

The overall time required for complete and successful development was prolonged at lower and higher salinities (35‰ and 10‰). At 35‰ only 17.5% developed normally, and it was completed in 18 days time; at 30‰, in 10 days; at 25‰ it was spread over 12 days; it was 15 days at 15‰–20‰; and 17 days at 10‰. The results are given graphically in Figure 1.

At 35‰ completion of the first stage required only 2 days, second stage 7 days and third 9 days. At 30‰ the first stage required 2 days, second and third stages required 4 days each. For the eggs kept at 25‰ the first stage required 2 days, second stage 6 days and third 4 days. Development at 20‰ and 15‰ was almost similar. The first stage required 2 days, second stage 6 days and third 7 days. At the lowest salinity tested only 17.5% developed into young

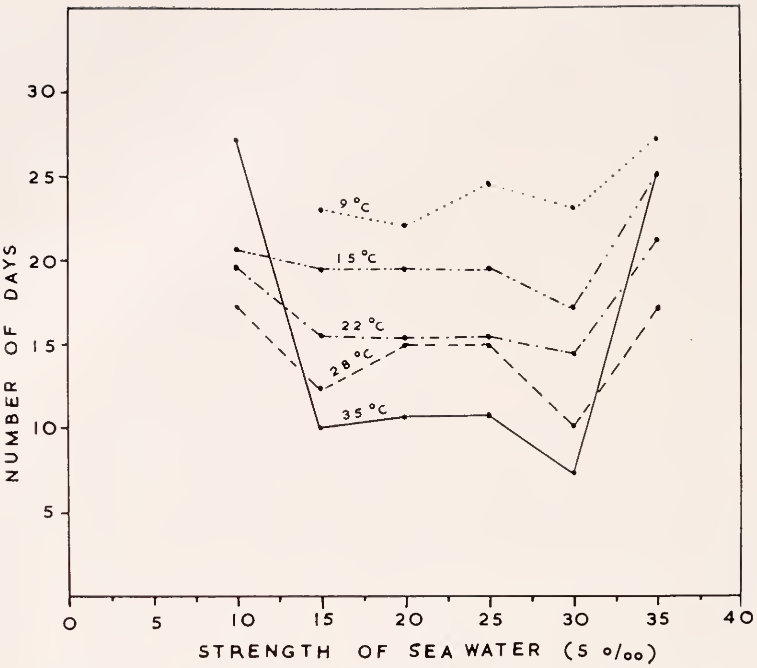

FIGURE 4. Combined effect of salinity and temperature on the incubation period of eggs of *Corophium triaenonyx* Stebbing.

ones. The first stage required 2 days, second stage 8 days and third 7 days. Results are presented graphically in Figure 2.

Observations on the combined effects of salinity and temperature revealed that 70% of the eggs developed into young ones with the dashlined boundary which was circumscribed by temperatures from 22° C to 28° C and salinity from 25‰ to 30‰. 99% was obtained at a temperature of 28° C and salinity 30‰ but this percentage was drastically reduced to 10%–13% at 10‰. At the lowest temperature tested (9° C), the percentage of development at all salinities was drastically reduced. The borderline appears to be 15° C in that a normal number of eggs (30%–40%) developed at 15‰, 20‰, and 25‰; 60% developed at 30‰; and 19.5%, at 35‰. Though development was hastened at 35° C between 15‰ and 30‰, at no salinity tested did 50% of the eggs develop into young ones. At 35‰ at none of the temperatures tested did as many as 50% of the eggs develop. Only a maximum of 40% was obtained at 28° C but this salinity was well above optimum and almost approached the upper limit of the embryonic development of *C. triaenonyx*. Results are given graphically in Figure 3.

At 9° C and 10‰ development ceased. A more detailed analysis of the data revealed that there was not much difference at 15° C and 22° C between 15‰ and 25‰. Development at 30‰ at all temperatures tested was dependent on temperature; that is, with an increase in temeprature the incubation period was reduced. At extreme salinities (10‰ and 35‰) the development was retarded at higher temperatures of 35° C. At 9° C at all salinities tested it was more than double the normal time. At 35° C between 15‰ and 30‰ the development time was between 7–11 days; but at 10‰ and 35‰, it was spread over 27 and 25 days, respectively. Results are presented graphically in Figure 4.

Two important points were noticed during the course of the experiments. First, the effect of salinity on various stages seems to be different. In all concentrations tested there was no variation in the time taken for the first stage (advanced gastrula stage) to be completed. Secondly, there is a relationship between the poor percentage of development and prolonged incubation periods.

DISCUSSION

The salinity tolerance in amphipods appeared to be different in different stages. Eggs of *Corophium triaenonyx* are found to develop at a fairly wide range of salinity, from 7.5‰ to 37.5‰, although the adults are able to tolerate a range between 0.6‰ to 59.8‰. Kinne (1953a) states that the eggs of *Gammarus duebeni* (near Kiel, Germany) can not develop below 1‰, though the adults live indefinitely in fresh water. The eggs of the shore crab *Carcinus maenas* develop normally only in salinities between 28‰ to 40‰, while adults can tolerate salinities below 4‰ (Broekhuysen, 1936). Similar findings have been reported in *Balanus* by Barnes (1953). It can be seen that although the eggs of *C. triaenonyx* developed through the above mentioned range, satisfactory percentage (70%) was obtained at a slightly smaller range of 20‰–32.5‰ and a maximum percentage at a still smaller range of 27.5‰–30‰. This was considered as the optimum range at a constant temperature of 28° C. According to Crisp and Costlow (1963) in *Balanus eburneus, Balanus amphitrite amphitrite* and *Chelonobia patula,* development took place at a normal rate at 25‰ to 40‰; but when they were exposed to salinities of 25‰–15‰ or 40‰–60‰, only a portion of the eggs hatched.

The normal development rate was 10–12 days between 25‰–30‰, but it was greatly prolonged (17–18 days) at extreme salinities (10‰ and 35‰). It is apparent from the results that extremes of salinities impose more limitations on development. Extreme salinities reduce the reproductive performance (Kinne, 1956b, 1956c, and 1958b). In England *Gammarus duebeni* of brackish-water lays more eggs than its counterpart in fresh water (Hynes, 1954). The optimal range of salinity for successful development in *Gammarus duebeni* seems to be 2‰–10‰ at a temperature of 8° C (Kinne, 1959). Kinne (1953a) reported that *Gammarus duebeni,* for which incubation period was determined at various salinities at 18° C–20° C, produced an average of 18 eggs in 2‰, 27 eggs at 10‰ and 17 eggs at 30‰. Vlasblom and Bolier (1971) stated that the incubation rate remained unaltered by changes in salinities but the juvenile number decreased.

Development ceases at 9° C at lower salinity of 10‰. The maximum percentage of development was noticed in salinities of 25‰–30‰ at temperatures 22° C–28° C. Fairly satisfactory development was observed at 20‰ and 15‰ at all temperatures. The incubation period was retarded at 35‰ and 10‰. According to Kinne (1953a) in *Gammarus duebeni* at 5° C and 6° C there was no significant difference, but above 8° C a progressive retardation was noticed both in 2‰ and 30‰ relative to the values observed at 10‰.

The lower limiting salinity for development in *C. triaenonyx* is 15‰ at 9° C and 10‰ between 15° C–35° C. According to Broekhuysen (1936) the lower limiting salinity for development of *Carcinus maenas* is about 20‰ at 16.3° C and 26‰ at 10° C. A close relationship between the effect of salinity on the rate of development and survival has been demonstrated by Costlow, Bookhout and Monroe (1960) in several brachyuran larvae.

It is clear that the distribution of *Corophium triaenonyx in* Visakhapatnam Harbor, and presumably in other brackish and estuarine habitats, will be limited by the effects of salinity and the combined effects of salinity and temperature. It should be possible to define in terms of the physical properties of the waters (ranges of salinity in relation to ranges of temperatures) which zones of harbors and estuaries will support stocks of *C. trianenoyx* capable of successful reproduction and therefore where these tube building amphipods are likely to become established as permanent elements of the local fauna.

I am grateful to Professor K. Hanumantha Rao, Head of the Department of Zoology, Andhra University for guidance and helpful criticism.

SUMMARY

1. Experiments were conducted to determine the percentage of development and incubation rate of eggs of *Corophium triaenonyx* at various salinities from 5‰ to 40‰.

2. The eggs develop only through a range of salinities from 7.5‰ to 37.5‰, although the adults are able to tolerate a range bebtween 0.6‰ to 59.8‰. A satisfactory percentage of development occurred between 20‰ and 32.5‰, but the optimum range was between 27.5‰ and 30‰ at a constant temperature of 28° C (± 0.1° C). Extreme salinities retarded the incubation period, being 18 and 17 days at 35‰ and 10‰, respectively.

3. In the experiments conducted to determine the combined effect of salinity and temperature, it was revealed that the best results were obtained at a combination of 30‰ and 28° C. The upper limiting salinity appeared to be 35‰ at all temperatures and lower limiting salinity is 10‰ between 15°C and 35° C and 15‰ at 9° C. High temperatures at extreme salinities retarded the development.

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THE LIFE CYCLES, INTERMEDIATE HOSTS, AND LARVAL STAGES OF *RHIPIDOCOTYLE TRANSVERSALE* CHANDLER, 1935 AND *RHIPIDOCOTYLE LINTONI* HOPKINS, 1954: LIFE-CYCLES AND SYSTEMATICS OF BUCEPHALID TREMATODES[1]

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Rhipidocotyle transversale was described by Chandler (1935) from metacercariae encysted in the muscles of silverside minnows, *Menidia menidia*, from Galveston Bay, Texas. *Rhipidocotyle lintoni* was described by Hopkins (1954) from mature specimens found in the intestine of gars, *Strongylura marina*, taken at Port Aransas, Texas, Grand Isle, Louisiana, and Gloucester Point, Virginia. These worms have been known for seventy-five years, but intially the species were confused, the descriptions were deficient, and the taxonomic allocations were erroneous. They had been included in the genus *Gasterostomum* by Linton in earlier publications.

Linton (1900) described and figured unidentified gasterostome trematodes from *Tylosurus marinus* (= *Strongylura marina*) and juvenile specimens encysted in *Menidia notata* at Woods Hole, Massachusetts. During the summers of 1901 and 1902, he studied parasites of fishes at the laboratory of the U. S. Bureau of Fisheries in Beaufort, North Carolina. Notes and sketches made there provided the basis for his report (Linton, 1905), in which he described gasterostomes from the intestines of a number of fishes under the name *Gasterostomum gracilescens* Rudolphi. Encysted metacercariae from *M. menidia* were assigned to the same species. The specific description from the 1901 notes reads, (p. 357), "numerous; fusiform, pale red to colorless; not much variation in size. One measured 1.4 mm in length and 0.56 mm in greatest breadth." The 1902 notation recorded, (p. 357), "Dimensions, life, compressed; length, 1.4; maximum breadth 0.65; transverse diameter, anterior sucker 0.33; mouth 0.10; ova 0.018 by 0.011." "Small, minutely spinose, of very variable shapes, translucent white to orange yellow, depending on relative abundance of ova. *The anterior end in some was curved ventrad, turning the sucker into a hood-like organ*" (italics added). After describing specimens from *S. marina*, Linton (1905) noted, p. 357, "This same species was found in this host at Woods Hole, Mass., but was not identified. (Bull. U. S. Fish Commission for 1899, pp. 277, 298, pl. XLI, Fig. 91, and p. 442, pl. XXXIV, Figs. 367, 368)". Figures 91 and 368 show an expanded lobe overhanging the sucker and can be identified as *R. transversale*. Linton (1905) reported *G. gracilescens* from other fishes; encysted metacercariae in the muscles of *M. menidia* and on the viscera of the striped anchovy, *Stolephorus brownii*;

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immature forms from the intestine of *Opsanus tau* and *Caranx hippos;* and mature worms from *Paralichthys albiguttatus* and *Pomatomus saltatrix*. The metacercarciae were almost full-grown in *M. menidia* and it is possible, indeed probable, that the specimens from hosts other than *S. marina,* may be the result of predation.

Gasterostome trematodes were reported from fishes of Bermuda by Linton (1907) and of Tortugas, Florida (Linton, 1910), but without significant new information. The descriptions are inadequate and the generic allocations are equivocal. A summary and review of his studies on trematodes of fishes mainly from the Woods Hole region was published by Linton (1940). In it, the species formerly described as *G. gracilescens* was transferred to *Prosorhynchus*. The specific description was revised, augmented, and illustrated by figures 245–249. Linton noted (p. 31), "In some there is a distinct cap overhanging the anterior sucker (pl. 18, Fig. 246). In others the cap is reduced to a buttonlike process, and in still others there is no trace of it. In some, also, the anterior sucker appears to be retracted (pl. 18, Fig. 247)."

Hopkins (1954) reported that the gasterostome trematodes from *S. marina,* described by Linton (1900, 1905, 1940) as *Proserhynchus gracilescens,* belong to two different species; one is identical with *Rhipidocotyle transversale* Chandler, 1935 and the other was described as a new species, *Rhipidoctyle lintoni*. The species with the hood-like lobe overhanging the anterior sucker was recognized as *R. transversale,* while *R. lintoni* had only a button-like lobe. Hopkins found gars, *S. marina,* infected with both species and *M. menidia* carrying metacercariae of *R. transversale* at Port Aransas, Texas; Grand Isle, Louisiana; and Gloucester Point, Virginia. Figures 245 and 248 in Linton (1940) represent specimens of *R. lintoni* and Figure 246 is of *R. transversale*. It is clear that Linton had both *R. transversale* and *R. lintoni,* and representatives of the two species were included in his description of *P. gracilescens*.

Linton (1900, 1905) had indicated the probable identity of the metacercariae encysted in *M. menidia* and the mature worms from the intestine of gars. This determination was confirmed by Hopkins (1954); he predicated that the metacercariae are specifically identical with *R. transversale,* but the metacercariae of *R. lintoni* were not recognized. Adults of both *R. transversale* and *R. lintoni* occur regularly in *S. marina* during the summer at Woods Hole and metacercariae (juveniles) of both species are common in *M. menidia* throughout the year. Both species may occur in one and the same fish, adults in gars and metacercariae in silversides. In distribution, the two species, *S. marina* and *M. menidia* extend from Cape Cod to Texas. So far as it is known, gasterostome trematodes utilize bivalve mollusks as first intermediate hosts. Accordingly, it is clear that certain bivalve species, which has the same distribution and occur in the same ecological milieu as the gars and silversides, must harbor the asexual generations of *R. transversale* and *R. lintoni*. A list of such mollusks was prepared and a systematic search was undertaken. The more common species, especially those of economic importance that have been extensively studied, could be excluded and efforts were concentrated on the small, inconspicuous clams. This procedure led to the discovery that *Lyonsia hyalina* harbors the larval stages of either or both *R. transversale* and *R. lintoni*. In a preliminary note, Stunkard (1974b) announced the first intermediate host.

The adult stages of *R. transversale* and *R. lintoni* occur in the same host-species, and both were described briefly by Hopkins (1954). The metacercariae of both develop in *M. menidia,* and it is probable that the sporocyst and cercarial generations of both species occur in *L. hyalina,* but present information is not adequate to distinguish between them. The sporocysts and cercariae do not manifest morphological features that can be used for specific determination and it is probable that biochemical methods must be employed to decide whether *L. hyalina* harbors one or two distinct species.

Material and Methods

Strongylura marina is a littoral species that frequently ascends rivers to considerable distances. It feeds on small fishes, especially *M. menidia.* According to Breder (1929), it is "known from Maine to Texas, but not common north of Cape Cod."

Menidia menidia is omnivorous; the fishes congregate in schools of similar-sized individuals. They frequent sandy and gravelly shores among clumps of sedge-grass (*Spartina* sp.) in inner bays and river-mouths and follow the tides a few meters from the water's edge. They are resident throughout the year and often run into brackish water. Formerly, two species of *Menidia* were recognized; *M. notata* in the area from Nova Scotia to Virginia and *M. menidia,* from Virginia to Texas; but the populations intergrade and specific distinctions are no longer acknowledged. The second intermediate host, *M. menidia,* carries natural infections of *R. transversale* and *R. lintoni* and provides developmental stages of both species, from recently penetrated metacercariae to encysted, full-grown juveniles. However, metacercariae of the two species were recognized only after development of the lobe overhanging the anterior sucker. This feature is difficult to evaluate since the lobe is muscular, mobile, and may appear and disappear with contractions of different sets of muscles.

Experimental infection of *M. menidia* was effected; large and small specimens were exposed to cercariae and later examination of the fishes disclosed details of penetration by the bodies of the cercariae after the tails had been discarded. A fish was exposed in a bowl at 10 AM, July 27, 1974, to several hundred cercariae. It was removed at noon and very few cercariae were left in the water, while hundreds of tails were found on the bottom of the bowl. Fishes exposed to massive infections died in 24 to 48 hours. Other fishes, exposed to large numbers of cercariae, were transferred to small holding tanks and fed bits of clams, *Mytilus edulis* and *Modiolus modiolus.* But *M. menidia* is an active species and difficult to maintain in captivity. Many of the fishes died, but dissection of those that survived for two to four weeks provided small worms of experimental infection that were identical with those of natural infection.

Lyonsia hyalina is the first intermediate host of either or both *R. transversale* and *R. lintoni.* Observations on living bivalves of New England, including *L. hyalina,* were recorded by Morse (1919); their morphology and ciliary mechanisms were described by Kellogg (1915); and the development of *L. hyalina* by Chanley and Castagna (1966). Individuals reach a length of 16 to 20 mm; the shell is thin, fragile, and the periostracum bears fine, filamentous processes which collect and hold a coat of fine sand grains. The edges of the mantle are fused ventrally; the siphons are separated, very short, and surrounded by rows of small, sensitive tentacles. The animals lie buried, with only the posterior end exposed and the siphons are retracted on the slightest stimulation. They occur in somewhat muddy rather than sandy bottom. Infections are common in certain areas and absent in others that superficially appear similar.

The clams were isolated, 10 in each bowl, with the water changed twice daily, to find those that were liberating cercariae. When cercariae appeared in a bowl, the clams were isolated singly to find the shedder. By changing the water periodically, the time of cercarial emergence could be detected. The cercariae are very small, transparent, hard to see, and the addition of a trace of neutral red to the sea water was helpful, since the stain accumulated in the glandular cells and intestines of the cercariae, and the color enabled an observer to pick them up more readily. The cercariae emerged intermittently during the day and the night, sometimes more heavily at one time than another, and it appears that light does not affect emergence. Data on incidence of infection may be misleading, since it may be 5% in one location and virtually absent not far away. As a rule, specimens less than 10 cm long are seldom infected and incidence increases with size and probable age of the mollusks. Most of the infected clams were taken from Bourne Pond, near Falmouth, Massachusetts. Dissection often disclosed mature infections in specimens from which no cercariae were released. To study sporocysts and developing cercariae, clams were dissected; the digestive glands and gonads were studied alive, after fixation and staining, and when cut in serial sections 10 microns in thickness.

When placed in bowls of sea water, specimens of *R. transversale* and *R. lintoni* shed enormous numbers of eggs. Eggs of the two species are the same size, 18 to 20 by 11 to 12 microns. Those in the terminal part of the uterus are embryonated when passed; the shells are thick, yellow in color and so opaque that details of the miracidium are not distinct. No ciliary movement was observed, but an occasional twitching of the larva was noted. The eggs are operculate but did not hatch in sea water although kept under observation for more than three weeks at summer temperature. Attempts to obtain miracidia by pressure on a coverglass or by changed osmotic conditions were not successful. The miracidium is quiescent and probably can live for a long time under favorable conditions. This feature must have survival value; if the larva emerged soon after liberation of the egg, its brief, ephemeral existence would afford slight change of entering the inhalent siphon of *L. hyalina*.

Attempts to infect *L. hyalina* by dropping eggs into the inhalent spihon were not productive. The clams were planted in mud in petri-dishes with the siphons exposed and placed in a current of slowly running sea water. Eggs were dropped in the water above the siphons; if the tentacles around the siphons were stimulated, they closed immediately and the eggs were lost in the excurrent streams from the dorsal siphon. It is doubtful whether or not any of the eggs actually entered the mantle cavity. Other clams were embedded in mud in a large, shallow, flat dish, covered with a coarse screen and left for three weeks in the bottom of a tank which contained eight *S. marina*. All the fishes yielded mature specimens of either or both *R. transversale* and *R. lintoni* when autopsied at the end of the summer. Three egg-shells with open opercula were found in the feces of clams that had been exposed to eggs, but primary sporocysts were not found on dissection of the clams two or three weeks later. Indeed, since the clams were recently collected, it would have been impossible to determine whether or not recent infections were natural or experimental unless massive infections were obtained. Since the eggs do not hatch in sea water, infection must be accomplished by ingestion of eggs and emergence of miracidia in the digestive tract of the mollusk. The primary sporocyst may remain in the tissue of the digestive gland, but recent infections, consisting of slender branches with small germ-balls but no cercariae, are present chiefly in the gonad. Growth and development of the sporocyst generations progressively destroy the gonad and then invade the digestive gland. Eventually, all of the host tissues become depleted and survival is doubtful.

Adult worms were studied alive and after fixation and staining. In small bowls, live gravid specimens of *R. transversale* and *R. lintoni* manifest differences in appearance and behavior. The anterior end of *R. transversale* is sticky and bits of debris adhere to it, but this condition was not observed with *R. lintoni*. *Rhipidocotyle lintoni* is more active; the worms migrated toward the edges of the bowl, whereas specimens of *R. transversale* remained near the center of the container. To obtain representative specimens, worms were fixed under slight coverglass pressure and good results were obtained also by the shaking method of Looss. With this method there is no flattening of specimens. In some, the genital atrium was everted with the protrusion of the genital lobes through the pore.

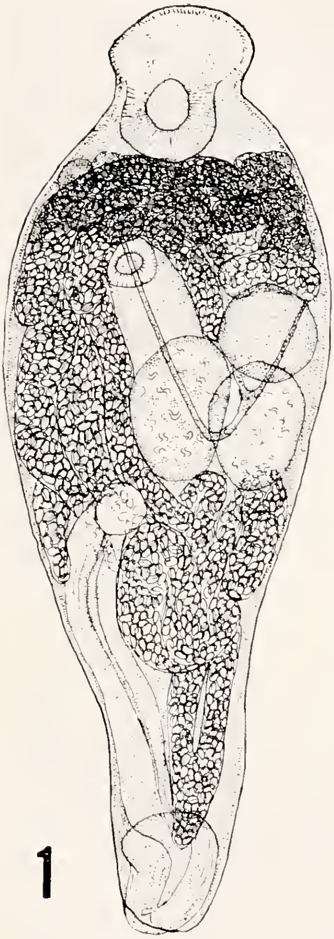
Existing descriptions of mature stages of *R. transversale* and *R. lintoni* are brief, incomplete, and present an unfortunate concept of the two species. They suggest a rigid, stereotyped structure; whereas, like all digenetic trematodes, the bucephalids are flexible, mobile, and highly variable worms. They manifest major changes in form and position of internal structures as different sets of muscles are contracted and relaxed. Mature worms from *S. marina,* when extended, may be narrow with almost parallel sides and three times as long as when contracted and expanded medially. With extension and retraction, there are corresponding changes in the relations of internal organs; the cirrus-sac may be some distance posterior to the gonads or almost contiguous with the anterior sucker, and there are resulting shifts in the arrangement of the coils of the uterus.

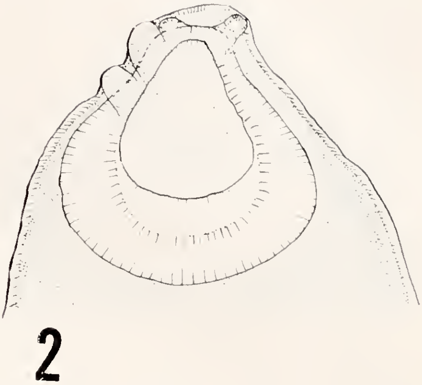
Examination of live material was facilitated by the use of vital dyes, especially neutral red. For permanent mounts, specimens were fixed in AFAG (alcohol, formalin, acetic acid, glycerol) or the solution of Duboscq-Brasil. Whole-mount preparations were stained with Mayer's paracarmine, Semichon's acetic carmine, and Ehrlich's acid haematoxylin. Sections were cut 10 microns in thickness which aids in computing measurements, stained with haematoxylin, and counterstained with erythrosin.

DESCRIPTIONS

Rhipidocotyle transversale (*adult stage*)

The description given by Hopkins (1954) is confirmed with additional information, especially on the reproductive systems. Fixed and stained gravid specimens (Fig. 1) measure 0.65 to 1.6 mm in length and 0.35 to 0.70 mm in width. Small specimens from the intestine of *S. marina,* 0.65 mm long and 0.35 mm wide may be





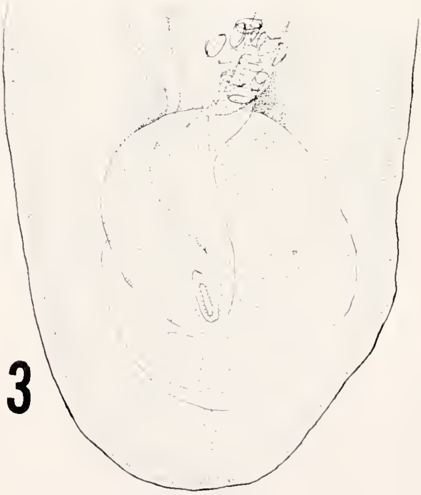


Figure 1. *R. transversale*, dorsal view of 1.38 mm long gravid specimen, fixed after shaking, attenuate posteriorly, genital atrium partially protruded.

Figure 2. *R. transversale*, ventral view, fixed under coverglass, showing anterior papillae.

Figure 3. *R. lintoni*, ventral view of genital atrium.

gravid while others that are much larger do not contain eggs. The worms may become virtually full-grown as metacercariae, and it appears that if small individuals are ingested, they may become mature in the final host without reaching full size. Although bilaterally symmetrical, the body is somewhat cylindrical, almost circular in cross-section. Typically, the worms are oval to ovate, wider anteriorly and often attenuate posteriorly. The hood which surmounts the anterior sucker is a mobile, muscular organ. Contracted, it may be almost spherical, but in flattened specimens it is oval, 0.15 to 0.22 mm wide and 0.08 to 0.12 mm long. The hood is variable in form; in living worms the anterior edge may be rounded and firm or it may manifest small papillae (Fig. 2), probably five in number, but not all are visible at the same time. Ducts from some 20 or more glandular cells, located in the area around and posterior to the sucker, pass forward and open at the anterior tip of the body. The contents of the ducts stain intensely with Semichon's technique. The secretion probably accounts for the adhesion of particles to the anterior end of living worms.

The tegument contains minute, closely set spines which decrease in size and number posteriorly. The body wall consists of circular, longitudinal and oblique muscle fibers, and the parenchyma is relatively loose and vacuolate.

The mouth is median, ventral, located near the posterior end of the anterior third of the body. The pharynx is spherical to barrel-shaped, usually flattened, 0.09 to 0.11 mm in diameter. There is a short esophagus, surrounded by glandular cells. The intestine is saccate, often with one or more peripheral pockets, and its dimensions depend on the amount of contained material.

The excretory pore is terminal and the vesicle extends forward on the dorsal side of the body to the level of the gonads. The collecting ducts, capillaries and flame-cells are obscured in gravid worms but may be traced in juvenile specimens. The collecting ducts open near the anterior end of the vesicle, and the flame-cell formula is $2[(2+2)+(2+2)]$, as described by Hopkins (1954).

Male reproductive organs. The testes are spherical to oval, 0.15 to 0.25 mm in diameter. They are situated obliquely on the right side, near or slightly posterior to midbody length, and may partially overlap each other and the ovary. Their positions vary from tandem to opposite with changes in the elongation and retraction of the body. In the specimen shown in Figure 1, the anterior testis is ventral in position, slightly mediad and posterior to the ovary. The posterior testis is dorsal and partially overlaps both the ovary and anterior testis. Usually the posterior testis is posterior to the ovary. In other specimens, the anterior testis is dorsal and the posterior testis is ventral in position. Sperm ducts arise from the medial faces of the testes and unite to form the vas deferens, which opens into the anterior end of a long, cylindrical organ, usually described as a cirrus-sac. The designation is questionable since the organ does not enclose a cirrus and subserves other functions. It is located on the left side of the body about midway between dorsal and ventral surfaces and extends from the level of the gonads to the genital atrium. It may be bent or slightly sigmoid and, in Figure 1 it is 0.38 mm long and 0.12 mm wide. The wall is composed of an external layer of strong longitudinal fibers, inserted near the ends of the sac, and an inner layer of delicate circular fibers. In the anterior end, the vas deferens expands to form an oval to spherical seminal vesicle, 0.09 to 0.12 mm in diameter. From the vesicle a short curved duct opens into a long canal, surrounded by cells of the prostate gland, which extends posteriad through the cirrus-sac. It enters the ventrolateral aspect of the genital atrium where it becomes a thin-walled ejaculatory duct that opens into the cavity of the atrium.

The distal end of the "cirrus-sac" is inserted in a circular manner on the anterior wall of the genital atrium, (Fig. 3), an oval to pyriform organ, 0.15 to

0.20 mm in diameter which receives the ejaculatory duct from the prostate gland and the metratermal end of the uterus. The wall of the atrium is composed of longitudinal and strong circular muscle fibers. From the atrium, a short duct leads to the gential pore, situated ventrally near the posterior end of the body. From the anterior wall of the atrium two unequal, curved genital lobes extend backward and partially surround a cavity which is continuous with the duct to the external pore. In the posterior wall of the atrium there is a cluster of glandular cells. In certain worms killed by the shaking method of Looss, the genital atrium is everted and the lobes are protruded through the genital pore. In Figure 1, one of the lobes extends slightly beyond the posterior end of the body. Odhner (1905) described the male organs of *Prosorhynchus squamatus* and other gasterostomes and their probable function. He suggested that the glandular cells in the atrium of *Prosorhynchus* spp. produce a secretion that encloses spermatozoa to form spermatophores. In *Gasterostomum* spp., he postulated that contraction of the powerful longitudinal muscles of the wall of the "cirrus-sac" could exert pressure on the atrium, causing distension of the genital lobes and their protrusion through the genital pore and transmission of spermatozoa. He stated, p. 303, "Dass der Geschlechtszapfen hier als Kopulationsorgan fungiert, ist also ganz deutlich; bei *Gast. gracilescens* (Rud.) habe ich auch mehrmals beobachtet, dass das Organ aus der äusseren Geschlechtsöffnung hinausgragt."

Female reproductive organs. The ovary is spherical to oval, 0.12 to 0.20 mm in diameter, situated dorsally on the right side, near the middle of body-length. The oviduct arises from the median posterior face; it passes posteriad and ventrad and shortly gives off the Laurer's canal which opens on the dorsal surface of the body. Where the canal joins the oviduct, there may be a slight enlargement filled with spermatozoa, but a definite seminal receptacle is not present. The oviduct then receives the duct from the vitelline receptacle and enters Mehlis' gland. The gland is situated in the interval between the ovary and the testes and is oval, 0.06 to 0.08 mm in diameter. The vitellaria consist of a band of follicles, each 0.032 to 0.046 mm in diameter, which extends across the dorsal side of the body a short distance posterior to the anterior sucker. The follicles of the two sides meet in the median plane; short ducts from the individual follicles unite to form two larger ducts that pass laterad on either side and at the ends of the follicles pass posteriad. The vitelline ducts unite to form a loop which passes posteriad to the level of the testes and gives off a short common vitelline duct that expands into the reservoir and communicates with the oviduct as noted previously. The uterus emerges from Mehlis' gland and makes a loop posteriad, then turns forward and passes to the level of the vitelline follicles where it crosses to the opposite side of the body. The uterus continues in loops and coils, filling all available space anterior and posterior to the digestive cecum. The final descending limb ends in a short metraterm which enters the dorsolateral face of the genital atrium and opens into the cavity of the atrium. Eggs in the initial part of the uterus are thin-shelled, flexible in shape. The shells are permeable and young embryos stain readily. In later portions of the uterus the eggs become hard-shelled, yellow in color and are not permeable to stains. Eggs measure 0.018 to 0.020 by 0.011 to 0.012 mm.

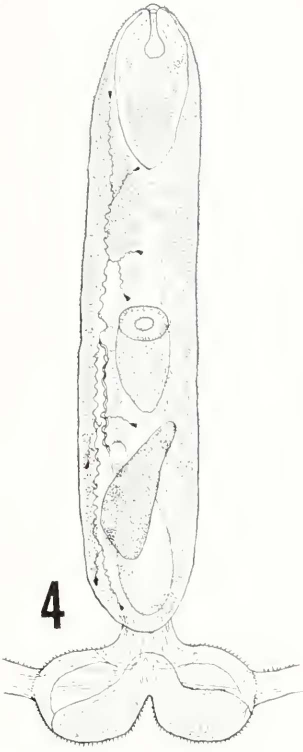
Rhipidocotyle lintoni (*adult stage*)

In the original description, Hopkins (1954) reported that *R. lintoni* is slightly smaller than *R. transversale* but that sizes of worms and of organs of the two species overlapped. The gland cells, so conspicuous around the anterior sucker of *R. transversale,* were not mentioned in the description of *R. lintoni*. The large hood or cowl, that surmounts the anterior sucker in *R. transversale* is represented in *R. lintoni* by a small cap. Hopkins noted differences in the shape and size of eggs of the two species; those of *R. lintoni* were slightly longer and narrower. His figure of *R. lintoni* shows a specimen with few eggs in the uterus, presumably a young worm, not fully mature.

Specimens of *R. transversale* and *R. lintoni* were abundant in the intestine of *S. marina* at Woods Hole, Massachusetts. Many mature *R. lintoni* are no larger than those reported by Hopkins; others are fully as large as those of *R. transversale*. The observations of Hopkins are confirmed, except for the size and shape of eggs. Examination of large numbers of eggs from *R. transversale* and *R. lintoni* did not reveal actual differences in shape or size. Except for differences in the hood and the absence of gland cells in the previtelline area of *R. lintoni,* the two species are so similar that a detailed description of *R. lintoni* would be a mere repetition of that of *R. transversale*.

Metacercariae

When the furcae of the cercaria make contact with a fish, they adhere firmly and their contraction brings the base of the tail-stem against the surface of the fish. Attachment by the base of the tail-stem provides a fulcrum from which the body of the cercaria attempts to enter the tissue of the host. The cercaria penetrates at the location of attachment. The cercariae will probably attack many kinds of fishes, but penetration and development apparently occur only in *M. menidia*. They may enter the gills, the wall of the mouth, around the eyes, and especially at the bases of the fins. On entry of the cercaria, the tail-stem and furcae are discarded and the body migrates by contraction of muscles in the body-wall and through vascular channels of the host. The cystogenous material was extruded early and after 24 to 48 hours, many of the worms were encysted. The worms encyst in muscles, even muscles to the eye-ball. The cyst-walls are thin, membranous and flexible. There was no visible reaction on the part of the host and no deposition of a second layer on the wall of the cyst. Immediately after encystment, the worms are smaller than the cercariae and do not completely fill the cysts which are oval to elongate. The cysts increase in size with development of the metacercariae and the largest measure 1.00 mm long and 0.50 mm wide. Since the metacercariae grow in their cysts, they must receive nourishment through the cyst-wall. Worms recovered two weeks after exposure showed little real growth. The intestine was enlarged and filled with oil droplets and cells of host-tissue, so the worms appeared larger and the largest, under cover-glass pressure measured 0.30 long and 0.09 mm wide. As noted, fish that received very heavy infections died in a day or two and most of the others were lost in the first two weeks. No specific differences were noted in the cercariae or in small metacercariae recovered four to six weeks after exposure. When the meta-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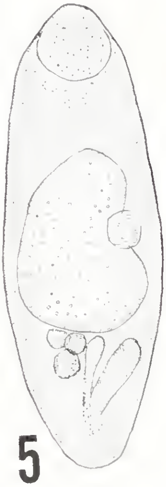
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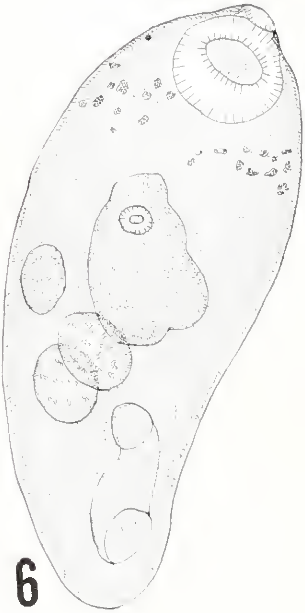
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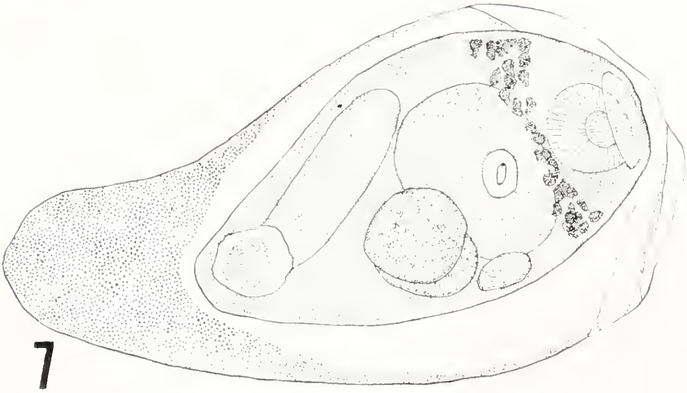
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FIGURE 4. *Rhipidocotyle* sp., Cercaria from *L. hyalina*, body and tail-stem (0.2 mm long); outline from fixed and stained specimen, details from living cercariae.

FIGURE 5. *Rhipidocotyle* sp., metacercaria at indifferent stage: shows regression of penetrantorium and formation of anterior sucker.

FIGURE 6. *R. lintoni*, metacercaria (alive, 0.5 mm), with early development of the vitellaria.

FIGURE 7. *R. transversale*, dorsal view, in cyst (1 mm long), vitellaria well formed.

cercaria is one-third to one-half grown, the anterior penetrating organ, designated the penetrantorium by Stunkard (1973) is undergoing regression and the anterior sucker is recognizable (Fig. 5). Only after transformation of the anterior ends and formation of the definitive lobes that surmount the anterior suckers can the two species be distinguished (Figs. 6 and 7).

Sporocysts

The sporocysts form tangled masses of colorless branching tubules that invade the gonad and digestive gland of *Lyonsia hyalina.* No miracidium-mother-sporocyst was found and since the sporocysts produce cercariae, presumably they should be regarded as daughter sporocysts. The tips of all branches are centers of rapid cell-multiplication. They advance by terminal growth into the haemocoelic spaces and ultimately destroy and replace the molluscan tissue (Figs. 8 and 9). On infection of the clam, the miracidium-mother-sporocyst, liberated in the intestine, enters the digestive gland. In mild and presumably recent infections, most of the sporocysts are in the gonad, which seems to be the seat of early involvement, but eventually both gonad and digestive gland are infiltrated and filled with tubules. The diameter of the tubules is variable; sections of very narrow width, 0.02 to 0.03 mm may alternate with short enlarged, distended sections as much as 0.13 mm in diameter. Often longer sections, 0.20 to 0.30 mm long and 0.07 to 0.10 mm in diameter, filled with developing cercariae, are followed by narrow stretches, containing a single row of small germ-balls. The walls of the sporocyst are capable of contraction and distension, but no movement was observed. In certain areas the tegument appears to be bounded by a membrane, lying over a single layer of circular muscle fibers and below this a layer of longitudinal fibers. In other areas, the external membrane appears to be separated from the muscle layers by a narrow band of cytoplasm that contains flattened nuclei. Below the muscle layers there are parenchymal cells and a fluid filled lumen. Certain of these cells have nuclei that stain heavily with haematoxylin and have little cytoplasm. They are more numerous near the tips of the tubules and may be germinal rather than somatic in nature. It appears that these cells undergo cleavage to form spherical germ-balls, 0.01 to 0.05 mm in diameter, composed of a few cells, which separate from the subtegmental layer and become free in the lumen of the tubule. Developmental stages from germ-balls to fully-formed cercariae fill the distended portions of the sporocyst.

Cercariae

In sporocysts, the germ-balls enlarge, become oval, and a constriction by circular muscles demarcates the bilobed tail. In a specimen 0.10 mm long and 0.03 mm wide, the tail stem is well formed and the furcae are about as long as the body. At this stage, they are curved medially and assume the characteristic crossed condition. Fully developed cercariae emerge from the haemocoele into the mantle cavity and pass to the exterior in the excurrent stream through the dorsal siphon. They have the specific gravity of sea water and hang from the furcae which extend upward. Retraction of the furcae, which proceeds in a spiral manner, raises the body and the cercariae are wafted about by currents in the water. They can not swim.

The body is oval to elongate with almost parallel sides, 0.12 to 0.25 mm in length and 0.03 to 0.07 mm in width (Fig. 4). It tends to be circular in cross-section when retracted and flattened on extension. The integument contains spines which decrease in size and number posteriorly. The body wall consists of circular, longitudinal and oblique muscle fibers. The parenchyma contains cystogenous glands, arranged in two longitudinal bands on each side of the body. The cells are irregular in size and shape.

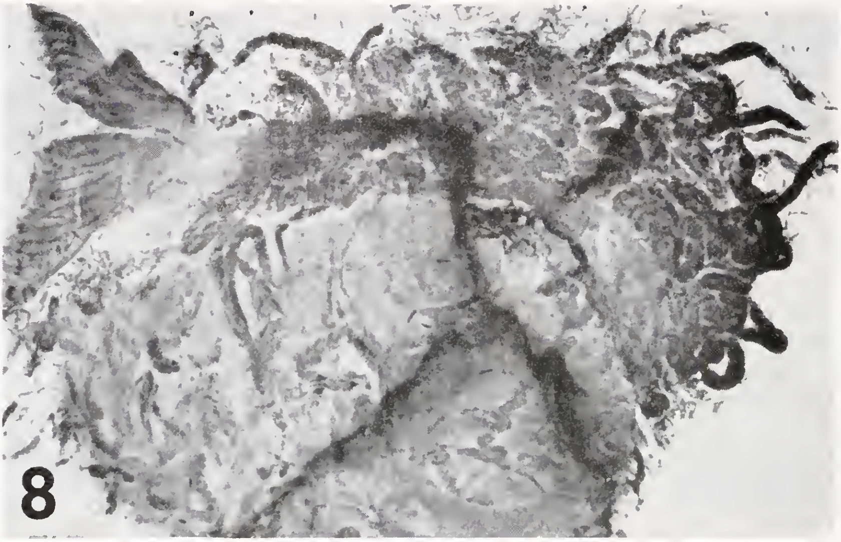
The tail-stem is 0.02 to 0.035 mm long and 0.04 to 0.07 mm wide. It is bilobed, indented posteriorly with a cup-like depression that may function in adhesion. The furcae arise from the anterolateral faces of the tail-stem; their bases are 0.009 to 0.012 mm wide and when fully extended, the furcae are filamentous, ten to fifteen times the length of the body. The furcae have strong circular and longitudinal muscles. The posterior, external surface of each bears papillae and adhesive glands by which they attach to the surface of a fish. When the furca is coiled, the adhesive surface is always external. The posterior half of the tail-stem is filled with similar adhesive glands and when immobilized, as when compressed by slight pressure from a coverglass, the body performs characteristic movements. First, it elongates to full extent, then snaps backward, right or left, until the anterior tip is at the level of the tail-stem, and then again fully extended. The movements occur at intervals of two to five seconds and appear to be the same as those employed in penetration of the skin of a fish.

The so-called "anterior organ" or "penetrating organ" of various authors is 0.05 to 0.06 mm long and 0.035 to 0.045 mm wide. Anteriorly, it opens to the surface by a subterminal pore. From the pore a short duct leads inward to a cupuliform expansion. The posterior portion of the organ is filled with penetration gland cells whose ducts pass forward and open into the cup-like expansion. The posterior half of the organ contains an enormous number of closely packed nuclei. It is suggested that they may be concerned with the formation of the anterior sucker after the regression of the anterior organ. In a discussion of similar "anterior organs" in strigeid and schistosome cercariae, Stunkard (1973) proposed that in these furcocercous cercariae, the organ that functions for penetration of the skin of the next host be designated a "penetrantorium" and the anterior organ of the bucephalid cercaria is a homologous structure.

The pharynx is located near the middle of the body and measures 0.011 to 0.013 mm in diameter. The intestine is mostly post-pharyngeal in extent and the contents stain with neutral red. As the reproductive organs mature, the digestive cecum is pushed forward.

The reproductive organs are represented by a curved mass of deeply staining germinal cells that extends posteriorly from the pharynx to almost the posterior end of the body (Fig. 4). It is the anlage of the gonads and the cirrus sac.

The excretory system begins as separate right and left components in the germ-ball stage with pores at the tips of the furcae. As development of the cercaria is completed, the ducts in the furcae are obliterated and secondary pores are formed at the anterolateral faces of the tail-stem. The loss of the tail-stem leaves a terminal excretory pore. The vesicle extends forward to the level of the gonads and the system is mesostomate with a flame-cell formula of $2[(2+2)+(2+2)]$.



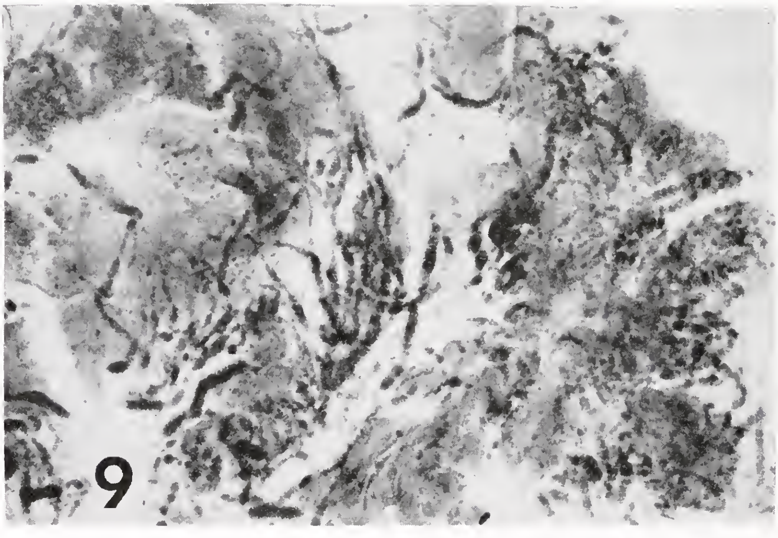


FIGURE 8. Pressed preparation of the tissues of the clam, *Lyonsia hyalina,* infiltrated with branching sporocysts of *Rhipidocotyle* sp.

FIGURE 9. Smear preparation of the tissues of the clam, *Lyonsia hyalina,* infiltrated with branching sporocysts of *Rhipidocotyle* sp.

TAXONOMY

The trematode family Bucephalidae

Prosorhynchoides gracilescens *new combination: validation of* Prosorhynchoides *Dollfus, 1929.* Rudolphi (1819) described three species of bucephalid worms from marine fishes: *Distoma gracilescens* from *Lophius piscatorius* taken at Trieste;

Monostomum galeatum from *Centronotus glaucus* and *Monostomum crucibulum* from *Muraena* spp., both taken at Naples. All were later transferred to *Gasterostomum*. In the period 1890 to 1900, encysted stages of gasterostome metacercariae were found in marine fishes, *Gadus* spp., *Phycis blennioides, Pleuronectes platessa, Belone belone,* and lampreys. These immature worms were generally referred to *G. gracilescens*. Odhner (1905) predicated that the ventral position of the mouth and saccate form of the intestine in gasterostome trematodes were primitive features, retained from a rhabdocoel ancestor, whereas the anterior location of the mouth in other digenetic species was a secondary character and the two groups were descended from different ancestral stocks. He erected two suborders: Gasterostomata for the species in which the mouth was ventral, and Prosostomata for those in which it was at or near the anterior end. Among the dozen or more species of *Gasterostomum* available to him, Odhner recognized two groups, separable on the structure of the adhesive organ, the location of the vitellaria, and the form of the excretory vesicle and of the copulatory organs. In one group the anterior end bore a simple or modified sucker and in the other the anterior end was provided with a solid rostellum or rhynchus. Members of the first group were referred to *Gasterostomum* von Siebold, 1848. Members of the second group were included in a new genus, *Prosorhynchus,* with *Prosorhynchus squamatus* n. sp., from *Cottus scorpius* as type species. Included in the new genus were *Prosorhynchus crucibulum* (Rudolphi, 1819) n. comb. from *Conger conger* and *Prosorhynchus aculcatus* n. sp. for specimens from *Conger conger* that had been assigned to *P. crucibulum* by van Beneden (1870). Odhner (1905) suggested that *Bucephalus cruz* Levinsen, 1881, a furcocercous cercaria from *Modiolaria discors,* may be the larval stage of *P. squamatus.*

The life-cycle of *P. squamatus* was reported by Chubrik (1952, 1966). It was confirmed by Matthews (1973a) who also described the morphology and life-history of *Prosorhynchus crucibulum*. Cercariae of both species developed in branching sporocysts in *Mytilus edulis* and metacercariae encysted in various fishes that served as second intermediate hosts. According to Shulman and Shulman-Albova (1953), *P. squamatus* occurs in several species of arctic fishes, and Chubrik (1966) reported progenetic gravid specimens in intermediate hosts. Matthews noted that measurements of the cercariae of *P. squamatus* agree with those given by Chubrik (1952) and with those given by Cole (1935) for the species from *M. edulis* described by him as *Cercaria mytili* n. sp. All have trilobed tail-stems and the description of *P. squamatus* by Matthews presents no item to differentiate it from *C. mytili*. It is apparent that the two descriptions pertain to the same species. Matthews found eight species of fish serve as second intermediate hosts of *P. crucibulum* but egg production occurred only in the intestine of *Conger conger.*

Prosorhynchus crucibulum is a boreal species which according to Brinkmann (1957) does not occur north of a line from Cape Cod through southern Iceland to Bronnoya, Norway, which is the northern limit of *C. conger*. Manter (1953) had noted differences between specimens of *P. squamatus* and *P. crucibulum* and predicated, p. 194, "In fact, *P. crucibulum* could well be considered in a different genus." Matthews (1973a) listed differences in the cercarial generations; in the morphology of the tail-stem, in shape of the excretory vesicle and in the flame-cell formula. These differences led Stunkard (1974a) to erect a new genus, *Rudolphinus,* with *Rudolphinus crucibulum* as the type species.

Diesing (1858) made a revision of the Trematoda. In it he erected the genus *Rhipidocotyle* to contain *Gasterostomum gracilescens* (Rudolphi, 1819) and *Gasterostomum minimum* Wagener, 1852, but he did not designate a type species. Stiles and Hassall (1908) tentatively selected *G. gracilescens* as type but Nicoll (1914) observed that the name *Rhipidocotyle* referred to a fan-shaped hood or cowl that surmounted the anterior sucker of *G. minimum,* but is not present in *G. gracilescens.* Accordingly, *Rhipidocotyle minima* was recognized as type of *Rhipidocotyle.* Nicoll (1914) adopted the family name Bucephalidae Poche, 1907 (syn. Gasterostomidae Braun, 1883) and erected two new subfamilies: Prosorhynchinae, with the single genus *Prosorhynchus* Odhner, 1905 and Bucephalinae with three genera: *Bucephalus* von Baer, 1826; *Bucephalopsis* Diesing, 1858; and Rhipidocotyle Diesing, 1858. The type material of *Gasterostemum galeatum* (Rudolphi, 1819) Stossich, 1858 and of *G. minimum* Wagener, 1852 from the Berlin Museum was studied by Eckmann (1932) who reported that *G. minimum* is identical with *Monostomum galeatum* Rudolphi, 1819. Consequently, *Rhipidocotyle galeata* (Rudolphi, 1819) Eckmann, 1932 is recognized as the type of the genus.

The species, *Distoma gracilescens* Rudolphi (1819 has had a resilient history; it was transferred to *Gasterostomum* by Wagener (1852), to *Rhipidocotyle* by Diesing (1858), to *Prosorhynchus* by Lebour (1912), to *Bucephalopsis* by Nicoll (1914), and to *Bucephaloides* by Hopkins (1954). According to Srivastava and Chauhan (1972), *Bucephaloides* Hopkins is a junior synonym of *Prosorhynchoides* Dollfus, 1929. Nagaty (1937) had recognized the validity of *Prosorhynchoides* Dollfus, 1929 with *Prosorhynchoides ovatus* (Linton, 1900) as type species. Srivastava and Chauhan (1972) transferred all the species previously included in *Bucephaloides* to *Prosorhynchoides. Distoma gracilescens* Rudolphi, 1819 thus becomes *Prosorhynchoides gracilescens* (Rudolphi, 1819) new combination.

The life-cycle of the species was reported, under the designation, *Bucephaloides gracilescens* (Rudolphi, 1819) Hopkins, 1954 by Matthews (1974). The sporocysts and cercariae were found in *Abra alba* from the Firth of Clyde, off the Isle Cumbrae, at depths of 40 fathoms and from the Lang channel at depths of 10 to 20 fathoms. About 15% of the clams were infected. Matthews reported, p. 3, "a thick solution of 'Polycel' was found useful in slowing down movements of cercariae and miracidia." This statement is surprising since gasterostome cercariae are relatively inactive and there was no other mention of the miracidium. Indeed, the miracidium has not been described for any marine gasterostome. Moreover, on p. 6 Matthews stated, "The flame cells divide as shown, the one posterior to the pharynx giving rise to that part of the flame system which passes into the posterior half of the body." And on p. 6, "Further division of the flame cells in *B. gracilescens* beyond the twelve flame cell stage have not been followed." These statements are obviously based on the unsupported presumption of Faust (1919), who accounted for the increase in flame-cells by division. During development of cercariae, additional flame-cells appear in a regular sequence and pattern, but not by division of previous cells. The cercaria is very similar and probably identical with *Cercaria syndosmyae* Lebour, 1912, described from the same host and the same location with an infection rate of about 20%. Matthews noted, p. 11, "The incidence and geographic location of this species is almost identical with that of *B. gracilescens* in this host." Matthews distinguished between the two species since Miss Lebour (1912) did not describe the sporocyst and in *C. syndosmyae* the figure shows the pharynx located at the level of the anterior half of the intestine. Neither of these feaures appears relevant. Miss Lebour figured only the tip of a branch of the sporocyst and the location of the pharynx with reference to the intestine is too variable to be species specific. The metacercaria has been recorded from nine different species of ganoid fishes. Its occurrence in the nervous system especially, encysted on cranial and spinal nerves of various fishes was noted by Munro (1785), and described by Olsson (1868), Johnstone (1905), and Lebour (1908, 1912). Matthews (1974) reported the development of the cercaria and the effect on the host. The metacercaria was linked with the adult on the basis of comparative morphology and ecology of hosts.

Rhipidocotyle campanula *(Dujardin, 1845) new combination: second freshwater bucephalid in Europe.* The taxonomy of the bucephalid trematodes is complicated because of unsupported and unwarranted presumptions concerning relations between larval and adult stages. Furcocercous cercariae from the European freshwater clams, *Anodonta mutabilis* and *Unio pictorum*, were described by von Baer (1827) as *Bucephalus polymorphus*. The larvae swim with the furcae in advance which prompted the generic name, *Bucephalus*. A graphic account of the swimming behavior was given by Badcock (1875) who reported, p. 142, that the cercariae "presented a beautiful sight of transparent creatures flying like eagles through the water, the wing-like appendages spreading out to an enormous length, and constantly in motion, with a general upward tendency. They never attached themselves to any object, but always swam freely, and were neither seen to feed upon other creatures, or to be objects of attack." Mature worms from the intestine of *Perca fluviatilis* and *Lucioperca* spp. were described by von Siebold (1848) as *Gasterostomum fimbriatum*. From morphological agreement, von Siebold predicated that these worms represented the adult stage of *B. polymorphus*. The probable identity was supported by Wagener (1858), Giard (1874), Ziegler (1883), and until recently all bucephalid parasites from European freshwater hosts were referred to a single species. Despite the recognized synonymy with *Bucephalus*, new species as described were assigned to *Gasterostomum* and a dozen or more were recognized by the end of the 19th century.

A new digenetic trematode was described by Dujardin (1845) under the name, *Distoma campanula*. He reported, p. 435, "J'ai trouvé à Rennes, dans l'intestin du brochet (*Esox lucius*) des petits distomes, moitié plus petits que le *Distoma nodulosum*, quoique adultes, et contenant aussi des oeufs plus petits de 0 mm 028; ils en différent en outre par leur ventouse ventral, beaucoup plus petite; la ventouse antèrieure est entourée à un large bord, finement plissé, en forme de cloche, mais non lobé; le tégument est parsemé de petites épines."

The species, *Distoma campanula* Dujardin, 1845 was redescribed and figured by Wedl (1858). The specimens were from the intestine of *Esox lucius*. The worms were about 1.00 mm long, 0.25 mm wide. The anterior sucker had two small lateral projections and was provided with a strong layer of muscle-fibers which extended in a concentric fashion on the ventral side and formed a ridge on the dorsal side of the anterior sucker. The pharynx was situated in the posterior half of the body and measured 0.06 mm in diameter. The vitellaria consisted of slight extensions in the anterior part of the body but were not shown in the figure. The uterus was massive, and filled with eggs that obscured the gonads. The eggs measured 0.028 mm in length. In the posterior part of the body Wedl figured an "oblonges Organ", apparently the cirrus-sac, but the genital pore was not observed. The tegument was provided with spines which diminished in size posteriorly. The redescription by Wedl clearly established the validity of *Distoma campanula* Dujardin.

Wagener (1852) reported *Gasterostomum fimbriatum* von Siebold, 1848 from the intestine of *Esox lucius* taken in the region of Berlin. He described the tentacles, which he found to number five, and observed that they may be extended and retracted. In the latter condition, they may not be visible. Additional descriptive data and figures were provided by Wagener in (1857). He (1858) suppressed *D. campanula* as a synonym of *G. fimbriatum* and recognized the identity of the latter species and *B. polymorphus*. The identity of *B. polymorphus* and *G. fimbriatum* was based on morphological similarity and the belief that there was only one species of bucephalid trematodes in the freshwater fishes of Europe. But there was no experimental evidence to support such a belief or for the identity of the sporocysts and cercariae that were found in the species of *Anodonta* and *Unio*. There was no assurance that *B. polymorphus* pertained to a single species. Furthermore, if *D. campanula* and *G. fimbriatum* are identical, the valid name of the species is *campanula*, not *fimbriatum*. However, later authors accepted the identity of the two forms and recognized *B. polymorphus* as the single bucephalid species in Europe.

Ziegler (1883) found mature bucephalid worms in the intestine of *Esox lucius* from the Ill River near Strassburg and sporocysts and cercariae in *Anodonta mutabilis*. The adults were described as *G. fimbriatum* and the cercariae as *B. polymorphus*. Specimens of *Leuciscus erythrophthalmus* were exposed to the cercariae and, on dissection later, harbored encysted metacercariae. The experiments lacked proper controls, but it is possible that Ziegler discovered the life-cycle of the species he studied. Discussing the cercariae, he declared that the anterior end does not bear a mouth as described by Pagenstecher (1857) but instead it consists of an "abgegrenster Komplex von Drüsen und Parenchymzellen (Fig. 9)". He referred the cercariae to *B. polymorphus* but noted in a footnote, p. 541, "Wenn Baer (No. 2, p. 575) grössere Dimensionen angiebt, so kann darin kaum specifischer Unterschied gesehen werden." Ziegler compared his adult specimens with descriptions of *G. fimbriatum* as given by Wagener (1852, 1857, 1858). He recalled that Wagener (1857) had described fingerlike processes at the anterior end of the body, "fünf gablige hohle Fühler oder Rüssel; sie könnten durch fünf musculöse Stränge eingestülpt werden, die von im Grunde des Saugnapfes verlaufenden scheiden umhüllt seien." Referring to the retractile tentacles, Ziegler stated, p. 542, "Ich habe dies Gebilde weder bei alten noch bei jungen Thieren je gesehen; da nun alle weiteren Angaben Wagener's vollkommen auf die mir vorliegende Form passen, so ist es mir unwahrscheinlich, dass seine

Darstellungen auf eine andere Species sich beziehen; ich bin eher geneigt zu glauben, dass die "Fimbrien" nur unter selten, vielleicht abnormen Verhältnissen auftreten und werde bei der Beschreibung des vorderen Saugnapfes die Entstehung derselben zu erklären suchen." Nothwithstanding this declaration, in a footnote on the same page, Ziegler predicated, "Wenn nachgewissen würde, dass die Fimbrien bei *Gasterostomum fimbriatum* eine normale und konstante Erscheinung sind, so müsste die im Flussgebiet der Ill gefundene und von mir beschriebene Form als eine neue Species angesehen werden und könnte *Gasterostomum illense* genannt werden." Although Ziegler recognized that the specimens from *E. lucius* might not be referrable to *G. fimbriatum,* in his description and figures he included them in that species.

Lühe (1909) described *B. polymorphus* von Baer and listed *G. fimbriatum* von Siebold as a synonym. The adult worms were from *Esox lucius, Perca fluviatilis, Lioperca lucioperca,* and *Lota lota;* the cercariae developed in various Unionidae. But his figure of *B. polymorphus* was clearly of the species described by Dujardin (1845), Wedl (1858) and Ziegler (1883). It can not be included in *Bucephalus* and clearly is a member of the genus *Rhipidocotyle.* Whether or not *Gasterostomum* is a synonym of *Bucephalus* is still uncertain.

The presence of a single bucephalid species in the freshwater hosts in Europe was accepted by Eckmann (1932), Nagaty (1937), Dawes (1946) and Hopkins (1954). But the existence of two distinct species is now recognized. Kowal (1949) found two species which he described as *Bucephalus polymorphus* and *Bucephalus markewitschi.* Vejnar (1956) identified the species without tentacles as *Rhipidocotyle ilense* (Ziegler, 1883) new combination. Kozicka (1959) admitted the identity of *Distoma campanula* Dujardin, 1845 and *Rhipidocotyle illense* (Ziegler, 1883) Vejnar, 1956. Yamaguti (1971) declared that *Rhipidocotyle illense* (Ziegler, 1883) Dyk, 1954 is a synonym of *Distoma campanula* Dujardin, 1845. The synonymy is well established and the species without tentacles is designated by its correct name, *Rhipidocotyle campanula* (Dujardin, 1845) new comb.

Lacaze-Duthiers (1854) described sporocysts and cercariae from the oyster, *Ostrea edulis,* taken at Mahon in the Balearis Isles and from *Cardium rusticum,* taken in l'étang du Thau near Cette on the south coast of France. There is no assurance that the parasites from the two hosts and two locations are specifically identical. Indeed, there is strong probability that two distinct species are involved. Environmental conditions at the two locations are very different. *Ostrea edulis,* the type host, is an oceanic species and the water at Mahon has high salinity; whereas at l'étang du Thau, the water had a reduced and varying salinity and an increased and varying temperature. The name *C. rusticum* has been suppressed as a synonym of *Cardium glaucus* Bruguiere, 1789 and as a subspecies of *Cardium edule.* There is a question concerning the identity of *C. edule.* Discussing Mediterranean species, Mars (1966) declared, p. 300, "Rappelons que nous n'avons jamais recontré l'espèce atlantique *Cardium edule,* ni sur le littoral, ni dans les gisements quarternaires, ni dans aucun étang de nos régions."

Referring to *B. haimeanus,* Matthews (1973b) observed, p. 341, "In addition to *Ostrea edulis* L., the type host, it has been found in eight other species of bivalves namely, *Cardium rusticum* L., by Lacaze-Duthiers (1854); *C. edule* L. by Huet (1888), Johnstone, (1905), Pelseneer (1906), Lebour (1907) and

James and Bowers (1967); *Spisula solida* (L.) by Huet (1888); *Venerupois decussata* (L.) and *V. pullastra* (Montagu) by Vaullegeard (1894); *V. aurea* (Gmelin) by Andreu (1949); *Abra alba* (Wood) by Pelseneer (1906) and *Crossostrea virginica* by Tennent (1905, 1906, 1909). All of these, with the exception of *C. virginica,* occur in Europe. *C. virginica* inhabits coastal waters off the east coast of North America." Lebour (1912) reported that in Britain *B. haimaeanus* has been observed only in the cockle, *Cardium edule*, but always in the vicinity of oyster beds. The fact that in the Plymouth area the parasite infected only cockles but not oysters in adjacent areas, is additional evidence that the species in *C. edule* is not *B. haimeanus* Lacaze-Duthiers. Matthews (1973b) reported the life-cycle of *B. haimeanus* from *C. edule*. He predicated, p. 348, "*B. haimeanus* is associated with the fauna of the estuarine habitat, the first intermediate host being *Cardium edule* and the second intermediate host *Pomatoschistus microns*." The cockles were taken from Dovey Estuary, Cardigan Bay, Wales. Metacercariae were obtained experimentally in *Pomatoschistus microns* and *Pleuronectes platessa*. The metacercaria was assigned to *Bucephalus minimus* Stossich, 1887 on the basis of comparative morphology and ecology of hosts. Adult specimens were described by Stossich (1887) from *Morone labrax* from the Mediterranean and by Nicoll (1914) from the same host taken in the Irish Sea. Matthews observed, p. 349, "In *B. haimeanus* the tentacles are much reduced in comparison with *B. polymorphus* suggesting an evolutionary link with the marine *Rhipidocotyle* through the estuarine habitat." The report by Matthews confirms the conviction that the parasite from *C. edule* is not *B. haimeanus*.

The first report of an American bucephalid was made by McCrady (1874) who described branching sporocysts and gasterostome cercariae from the oyster, *Crossostrea viriginica* at Charleston, South Carolina. The sporocysts were rigid, not contractile, and apparently resembled those of *B. polymorphus* von Baer, 1827, a freshwater species in Europe rather than those of *B. haimeanus* Lacaze-Duthiers, 1854, a parasite of the European oyster. The furcae were long and provided with pointed cells along their length. The parasite was described as a new species, *Bucephalus cuculus.* Bucephalid sporocysts and cercariae were reported from New Jersey by Nelson (1890, 1903, 1915). *Bucephalus cuculus* was reported from oysters at the Gulf Biological Station, Cameron, Louisiana by Glaser (1904). Tennent (1905, 1906, 1909) identified the parasite of oysters at Charleston, S. Carolina as *B. haimeanus.* He presumed that the cercariae were identical with the metacercariae in *M. menidia* and the adults from *Strongylura marina* that had been assigned to *Gasterostomum gracilescens* by Linton (1905). None of these allocations were correct. The life-cycle had not been demonstrated for any American marine gasterostome until the report by Stunkard (1974b).

The life-cycles of three North American freshwater bucephalids have been elucidated. They are *Rhipidocotyle papillosa* (Woodhead, 1929) Eckmann, 1932 by Woodhead (1929); *Bucephalus elegans* Woodhead, 1929 by Woodhead (1930); and *Rhipidocotyle septpapillata* Krull, 1934 by Krull (1934) and Kniskern (1952a, b).

The other experimentally-proved life-cycle was done by Howell (1966). He showed that sporocyst and cercarial generations of *Bucephalus longicornutus* (Manter, 1954) occur in the New Zealand mud-oyster, *Ostrea lutaris* Hutton,

1873. Metacercariae were recovered from *Tripteron* spp. and *Acanthoclinus quadridactylus.* Adults occur in *Scorpaena cardinalis* Richardson.

At present, a challenging problem in the family Bucephalidae is the discovery of the life-cycles, secondary and definitive hosts of *B. haimeanus,* from the European oyster and of *B. cuculus* from the American oyster. It is distressing to realize that this situation has remained unresolved for more than a century.

SUMMARY

The life-cycles of *Rhipidocotyle transversale* Chandler, 1935 and *Rhipidoctoyle lintoni* Hopkins, 1954 have been determined. Both species employ the same intermediate and final hosts; metacercariae in silversides, *Menidia menidia,* and adults in gars, *Stronglyura marina.* Branching sporocysts and furcocercous cercariae occupy the gonad and digestive gland of *Lyonsia hyalina* but can not be assigned with certainty to either or both species, because identifying morphological features are not yet developed. Adult and metacercarial stages of both parasites occur in the same host-species and it is probable that the larval stages of both are in *L. hyalina.*

Recent studies on life-cycles of bucephalid trematodes demand revision of certain taxonomic allocations. The status of *Distoma gracilescens, Monostomum galeatum,* and *Monostomum crucibulum,* all described by Rudolphi (1819), are reviewed. *Distoma gracilescens* was transferred to *Gasterostomum* by Wagener (1852), to *Rhipidocotyle* by Diesing (1858), to *Prosorhynchus* by Lebour (1912), to *Bucephalopsis* by Nicoll (1914), and to *Bucephaloides* by Hopkins (1954). But *Bucephaloides* Hopkins, 1954 is a junior synonym of *Prosorhynchoides* Dollfus, 1929 and *Distoma gracilescens* becomes *Prosorhynchoides gracilescens* (Rudolphi, 1819) new combination. The life-cycle of the species was reported by Matthews (1974). *Monostomum galeatum* was transferred to *Gasterostomum* by Stossich (1898) and declared identical with *Gasterostomum minimum* Wagener, 1852 by Eckmann (1932). Accordingly, it becomes the type-species of *Rhipidocotyle* Diesing, 1858. *Monostomum crucibulum* was transferred to *Gasterostomum* by Gervais and Beneden (1859), and to *Prosorhynchus* by Odhner (1905). The life-cycle was reported by Matthews (1973a), and Stunkard (1974a) named it type of a new genus, *Rudolphinus.*

The status of *Bucephalus polyphemus* von Baer, 1827 and *Gasterostomum fimbriatum* von Siebold, 1848, long regarded as specifically identical and the only bucephalid species in freshwater hosts in Europe, is equivocal. *Bucephalus polymorphus* is the name of a cercaria whose adult stage is yet to be disclosed and the larval stages of *G. fimbriatum* are unknown.

Dujardin (1845) described small distomes from the intestine of *Esox lucius* taken at Rennes, France, as *Distoma campanula.* The species was redescribed and figured by Wedl (1858). *Distomum fimbriatum* von Siebold (1848) was redescribed by Wagener (1852, 1857, 1859). He (1858) suppressed *D. campanula* as a synonym of *G. fibriatum,* and recognized the latter species as the adult of *B. polymorphus.* Ziegler (1883) described mature bucephalid worms from *E. lucius* taken in the Ill River near Strassburg as *G. fimbriatum* and larval stages from *Anodonta mutabilis* as *B. polymorphus.* In a footnote, he predicated that if his specimens did not prove to be identical with *G. fimbriatum,* they should be regarded as a new species, *Gasterostomum illense.* A second bucephalid species from freshwater fishes in Europe was described by Kowal (1949), Vejnar (1956), and Kozicka (1959). It was designated *Rhipidocotyle illense* (Ziegler, 1883) Vejnar, 1956 by Kozicka (1959). Comparison with descriptions of *Distoma campanula* show such agreement that the two must be regarded as identical and the name of the species is *Rhipidocotyle campanula* (Dujardin, 1845) new combination.

Bucephalus haimeanus was described by Lacaze-Duthiers (1845) from cercariae found in oysters. *Ostrea edulis,* at Mahon in the Balearic Isles and in *Cardium edule* taken in l'étang du Thau near Cette on the south coast of France. There is no evidence that the cercariae from the two hosts and two locations are identical. Lebour (1912) reported *Cercaria haimeanus* from *C. edule* at Plymouth, but no infection in oysters. Matthews reported that cercariae from *C. edule,* identified as *Cercaria haimeanus,* developed into adults identical with *Bucephalus minimum* Stossich, 1887. The adult stages of the cercariate from European oysters, and of another species, *Bucephalus cuculus,* parasitic in the American oyster, *Crossostrea virginica,* are still unknown after more than one-hundred years.

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EXPERIMENTAL CULTURE OF THE ESTUARINE ECTOPROCT *CONOPEUM TENUISSIMUM* FROM CHESAPEAKE BAY[1]

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Virtually all of the 3500 extant species of ectoprocts feed by filtering plankton. But in spite of the fact that they are common components of both marine and estuarine suspension-feeding communities, little is known about the kinds of particles which are utilized by ectoprocts as food.

Gut contents of ectoprocts were described by Hentschel (1922) for *Sargassum*-encrusting *Membranipora tuberculata,* and by Hunt (1925) for two species from the Plymouth fishing grounds. The gut of *Membranipora tuberculata* held diatoms, coccolithophores, peridinian dinoflagellates, and *Physalia* nematocysts. The two Plymouth area ectoprocts contained small diatoms, silicoflagellates, peridinians, coccolithophores, algal cysts and detritus. Hunt also observed the capture of small flagellates by ectoprocts and speculated that they might serve as food sources.

Literature on the culture of marine ectoprocts has been scarce until recently. Hasper (1912) fed young colonies of *Bowerbankia pustulosa* on the diatoms *Nitzschia closterium forma minuta* (=*Phaedactylum tricornutum?*) and the cyanophyte *Pleurococcus mucosus.* Schneider (1959, 1963) cultivated *Bugula avicularia* on the colorless dinoflagellate *Oxyrrhis marina.*

Bullivant (1967, 1968) found that colonies of the ctenostome *Zoobotryon verticillatum* grew well on the chrysophyte flagellate *Monochrysis lutheri* and the aberrant diatom *Cyclotella nana* and the coccolithophore *Criocosphaera carterae,* but could not grow on the dinoflagellate *Amphidinium carterae,* the green flagellate *Dunaliella tertiolecta,* or the diatom *Thalassiosira fluviatilis.* Further experiments showed that colonies of the cheilostome *Bugula neritina* grew well on *Monochrysis,* but not on *Phaeodactylum.*

Recently Jebram (1968) has used *Oxyrrhis marina* (fed upon *Dunaliella*) and *Cryptomonas* sp. to culture several ectoproct species including *Alcyonidium* sp., *Bowerbankia gracilis, Farella repens, Electra crustulenta, E. monostachys, Conopeum seurati, C. reticulum* and *Bugula stolonifera.* Menon (1972) has also used *Cryptomonas* sp. to maintain colonies of *Electra pilosa, Conopeum reticulum* and *Membranipora membranacea* for experimental work.

The existing literature seems to suggest then, that each ectoproct species may react differently to each of a variety of food species. The factors responsible for such differential growth responses are unknown and require examination.

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This report examines the growth reactions of one species of ectoproct fed a variety of foods. The purpose of this experimentation is to identify some of the factors which may be responsible for differential growth responses and to examine the role of nutrition as it affects colony morphology.

The estuarine cheilostome *Conopeum tenuissimum* was chosen as the ectoproct to be used for the series of culture experiments. This species was common in the research area and is an important component of estuarine fouling communities along the east coast of the United States from Maine to Florida.

METHODS

Conopeum colonies used in the culture experiments were collected in the York River at the Virginia Institute of Marine Science (VIMS), Gloucester Point, Virginia. Artificial substrates, *i.e.*, bryozoan traps, were suspended from the VIMS pier for several days. The glass slide substrates were then removed and examined for *Conopeum* colonies. When one was located near the center of a slide it was isolated by careful cleaning and removal of all other organisms on the slide.

The size of the experimental colonies at the start of the culturing period ranged from 2–28 zooids. Six to eight colonies were grown on each experimental food medium.

The slides bearing the colonies were placed in rectangular pyrex dishes capable of holding one liter of water. Slides rested on the bottom or against the sides of the dishes so that expanded polypides were in either an upright or a horizontal position. The colonies were cleaned and rotated in position in the dish each time the feeding medium was changed. It was assumed that the position of colonies had no effect on growth as long as there was no build up of debris. This is indicated in nature by the occurrence of colonies on all surfaces of eelgrass and other substrates.

The experiments had been planned to begin in late June, 1972. At this time the Virginia coast and Chesapeake Bay were struck by the rains and flooding associated with tropical storm Agnes. This flooding caused salinities over the entire Bay to drop drastically. At Gloucester Point, Virginia where salinities in late June are usually around 16–17‰, the salinity dropped from 15.8‰ on the 23rd of June to 8.8‰ on the 28th.

It appeared that it might be several months before the York River salinity reached its pre-Agnes level, and it was feared that this low salinity might affect growth. Thus it was decided to raise the colonies on York River water mixed with enough water obtained from the Eastern Shore of Virginia (marine) to reach a salinity of about 16‰. This mixture was filtered through the laboratory filter system. Water was changed three times weekly, and tanks were cleaned and colonies fed at the same time. Temperatures for the 1972 experimental period ranged from 23.0–26.5° C and salinities from 15.8–17.5‰. Each of the media used to culture the *Conopeum* colonies is described below.

The chrysophyte flagellate *Monochrysis lutheri* (5.4 μm) was chosen as the first experimental food because it had been found to promote growth in numerous other invertebrate species including oyster larvae.

Dunaliella tertiolecta (6×9 μm), a chlorophyte flagellate, has also been utilized in culturing experiments, but reports of its food value have varied.

Va-12 is an unidentified chrysophate flagellate isolated from a "red tide" in the York River. Cells of this species average 4.5 × 4.2 μm in size; the shape is slightly longer than broad, the apical end somewhat smaller than the posterior end; some cells appear slightly concave on one side. This species was tried because oyster larvae being cultured at VIMS had shown good growth when Va-12 was used in combination with other foods.

Nannochloris occulata is a nonmotile chlorophyte only 2–3 μm size. This small species has also been used successfully in oyster larviculture at VIMS.

Cyclotella nana (6.4 μm), a small centric diatom with a siliceous cell wall, was chosen because of its size and because it had been utilized in the culture of other invertebrates.

Gymnodinium simplex is an unarmored dinoflagellate 6 × 8 μm in size. Because dinoflagellates are often an important component of nearshore and estuarine phytoplankton, I decided to try to culture *Conopeum* on species that would be found in its natural habitat. *Gymnodinium simplex*, while never a dominant in the York River waters, occurs with some frequency (MacKiernan, 1968) throughout the year.

Anacystis marinus is a small (1–2 μm) rounded species of cyanophyte. In the inner parts of the bay, blue-green algae are often of considerable importance. Therefore, it seemed desirable to test the value of a member of this group as a food for *Conopeum* colonies. While various workers have attempted to culture freshwater organisms on blue-green algae, there is no information on their use as a food for marine or estuarine invertebrates.

Because other invertebrates had been shown to grow better on a combination of foods than on a single algal food, two mixtures of foods were tried also: *Monochrysis-Dunaliella*, and *Monochrysis-Dunaliella-Cyclotella*.

In preliminary experiments during the previous year, oyster tank detritus had failed to support growth of *Conopeum* colonies, so it was decided to try feeding colonies directly with a culture of bacteria which had been isolated from the sides of the oyster tanks at VIMS. This bacterium has not been positively identified but is probably a pseudomonad or *Vibrio* species (S. Rivkin, VIMS, personal communication).

In addition to the culture of *Conopeum* on the different diets listed above, colonies were also cultured on five different concentrations of *Monochrysis* and also five concentrations of *Dunaliella*.

The algal cultures used were obtained from Dr. Franklyn D. Ott of the VIMS algal culture lab. The cultures were grown in large aerated glass vessels with constant illumination provided. *Nannochloris* was cultured at room temperature; the other species, at 16° C ± 4° C. The medium used in culturing the algae consisted of filtered York River sea water enriched by a stock nutrient mineral medium.

The algae were filtered through a 50 μm filter to remove any undissolved particles of medium, etc., and added to one liter of York River water which had been passed through a sand filter and then through two 1.0 μm Cuno cotton filters. At the time the medium was renewed, the colonies were carefully cleaned with a soft brush and the dishes were scrubbed clean of any detritus, dead algae, and fecal material that might have settled.

At each feeding the following quantities of algal medium were used: (1) for single foods, 60 ml of algal suspension; (2) for the two-food mixture, 30 ml of each algal suspension; (3) for the three-food mixture, 20 ml of each algal suspension; (4) for the first control, 60 ml of algal medium only; (5) for the bacteria, one pipetteful of bacteria; and (6) for colonies being given the various concentrations of *Monochrysis* or *Dunaliella,* 15, 30, 60, 90, or 120 ml/liters of sea water at each feeding.

Although the numbers of algae present per ml of culture suspension varied both according to species and according to stage in the growth of the algal population cycle, it was desirable to have an estimate of the quantities being fed to the ectoprocts. To facilitate estimation of food concentration each time the algal food suspensions were obtained a few ml were removed, the cells killed with a drop of formalin and immediately counted under the microscope using a hemocytometer.

The experiments ran for about 40 to 42 days for two reasons. First, by this time reproduction would have occurred in nature, and it was desired to see if some foods supported reproductive activity; and secondly, by this time the colonies could grow to the edges of the slides and thus nullify further quantitative growth records.

The growth of colonies was recorded by photography at least five times during the experimental period: at the start, at the finish and at three other times during the six week growth period. The few exceptions were due to photographs lost in processing, and the reduced photography of series exhibiting very little growth.

The photographs were arranged to prove a sequential picture of the changes in size and shape of each colony as it grew. Measurements were made of the number of zooids and number of generations (zooids in a direct line out from the ancestrula). These data were used to construct both growth curves for individual colonies and mean growth curves for all colonies receiving a particular diet.

RESULTS

General patterns of colony growth

In the initial four to six days of culture all young colonies of *Conopeum* increased zooid numbers at an exponential rate. This response is independent of diet as even starved colonies exhibited such initial growth. After this initial growth period both zooid number and colony growth pattern were found to be diet dependent. Each food treatment produced consistent characteristics of colony growth. The best single species food, *Dunaliella,* produced a healthy roundish colony similar in shape to colonies observed in nature (Fig. 1A, B). In contrast, a poor food such as *Cyclotella* produced a colony response typified by radiating biserial chains with few zooids (Fig. 1D). Most of the zooids present were heavily calcified and lacked functional polypides.

Growth responses to single species foods

Growth of *Conopeum* colonies was quantified by direct zooid counts. Table I and Figures 2 and 4 summarize and contrast the responses of the colonies to various foods. From Table I and Figure 2A it is evident that *Dunaliella* and *Gymnodinium* produced the best growth of all single foods tested. *Dunaliella-*

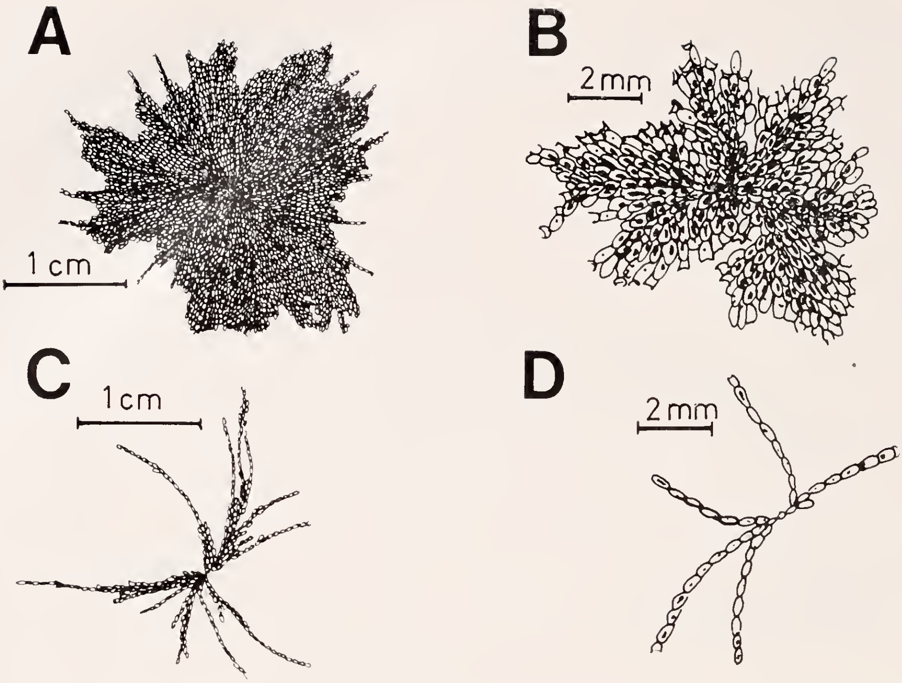

FIGURE 1. Examples of variation in growth form among *Conopeum tenuissimum* colonies raised on different foods. (A) Example of a colony raised on a good food, the chlorophyte flagellate *Dunaliella tertiolecta*, after 40 days of growth. Note the large number of zooids and almost circular shape of the colony due to distolateral budding to fill in the space between the major branches. (B) Morphology of a colony grown in the natural York River environment, after 20 days of growth. Dark coloration is due to food in the gut of polypides. Polypides in the outermost rows of zooids are still developing and have not yet begun to feed. (C) Example of a colony raised on a fair food, the chrysophyte flagellate *Monochrysis lutheri*, after 42 days of growth. Colony shows typical biradiate, branching shape, due to budding along major growth axes only. (D) Example of a colony raised on a poor food, the diatom *Cyclotella nana*, after 41 days of growth. Colony has small number of zooids, arranged in six branches. Most zooids are lacking polypides and are heavily calcified.

fed colonies (7/15–8/26; 42 days) exhibited the healthy circular form (Fig. 1A). At the end of the 42-day culture period all colonies appeared healthy. Observation of polypides showed some with intertentacular organs for release of eggs; these zooids contained ovaries with developing eggs. Other zooids had regenerating polypides and developing ovaries.

Gymnodinium was also a good food for *Conopeum* (Fig. 2A). The *Gymnodinium*-fed colonies (7/15–8/19; 8/21–10/2; 42 days) always had a pinkish or orangeish tinge due to the dinoflagellates in their guts. This unique coloration was the same as that observed in colonies taken from the York River, substantiating the idea that this species might naturally feed upon suitably sized dinoflagellates. It was not possible to examine the total growth response of *Conopeum* to *Gymnodinium*, as a failure in the VIMS algal culture system eleven

TABLE I

Ratio of mean number of zooids to mean number of generations in Conopeum colonies after six weeks of culture. A generation is the longest chain of zooids in a direct line outward from the ancestrula, usually measured on primary (distal) chain.

Food used	Number of colonies	Mean number of generations per colony	Mean number of zooids per colony	Ratio of zooid number to generation number
Mono-Dun-Cyclo	6	36	1629	45:1
Dun-Mono	8	29	1291	45:1
Dunaliella	7	33	2484	75:1
Gymnodinium	4	22	1409	64:1
Monochrysis	5	40	370	9.3:1
Va-12	7	31	201	6.5:1
Anacystis	7	12	48	4:1
Cyclotella	8	10	35	3.5:1
Nannochloris	5	15	42	2.8:1
Bacteria	7	7	16	2.3:1
Control (algal medium)	6	11	34	3.1:1
Control (sea water only)	5	8	21	2.6:1

days after commencement of the culture run killed the *Gymnodinium* culture. The colonies initially responded with rapid growth similar to that of *Dunaliella*-fed colonies. As the quality of the *Gymnodinium* decreased, the more or less even outward growth stopped and colonies begun producing radiating biserial chains. Once the algal culture was dead colony growth gradually ceased. This culture was repeated (8/21 to 10/2) and again the algal culture fluctuated in concentration and vigor. While the food supply was declining, the growth rate of the ectoprocts decreased, with many of the polypides in the central portions of the colonies degenerating. Once the food started to increase in density, the rate of growth of the colonies also increased.

At the end of the culture period two of the four cultured colonies contained some zooids full of sperm as well as some with developing eggs and polypides with intertentacular organs. Two other colonies, both smaller than those above, showed no evidence of reproductive activity.

Foods producing fair growth of *Conopeum* were *Monochrysis* (7/15–8/26; 42 days) and Va-12 (Fig. 2C). Both foods produced colonies of intermediate form (Fig. 1C). Zooid production was considerably less than for *Dunaliella*-fed colonies (Table 1) and neither food supported production of polypides with intertentacular organs or other reproductive structures.

The foods producing poor *Conopeum* growth were *Nannochloris, Cyclotella, Anacystis* and bacteria. The *Nannochloris*-fed culture (7/15–8/25; 41 days) produced a mean growth curve (Fig. 2D) very different from the growth on the other chlorophyte species *Dunaliella*. At the end of the culture period one colony was broken and appeared to be decaying. Among the other colonies, some had broken zooids while a few appeared to have functional polypides and were producing occasional new buds.

Conopeum cultured on *Cyclotella nana* (7/15–8/25; 41 days) appeared in poor condition. At the end of the culture period there were functioning polypides only

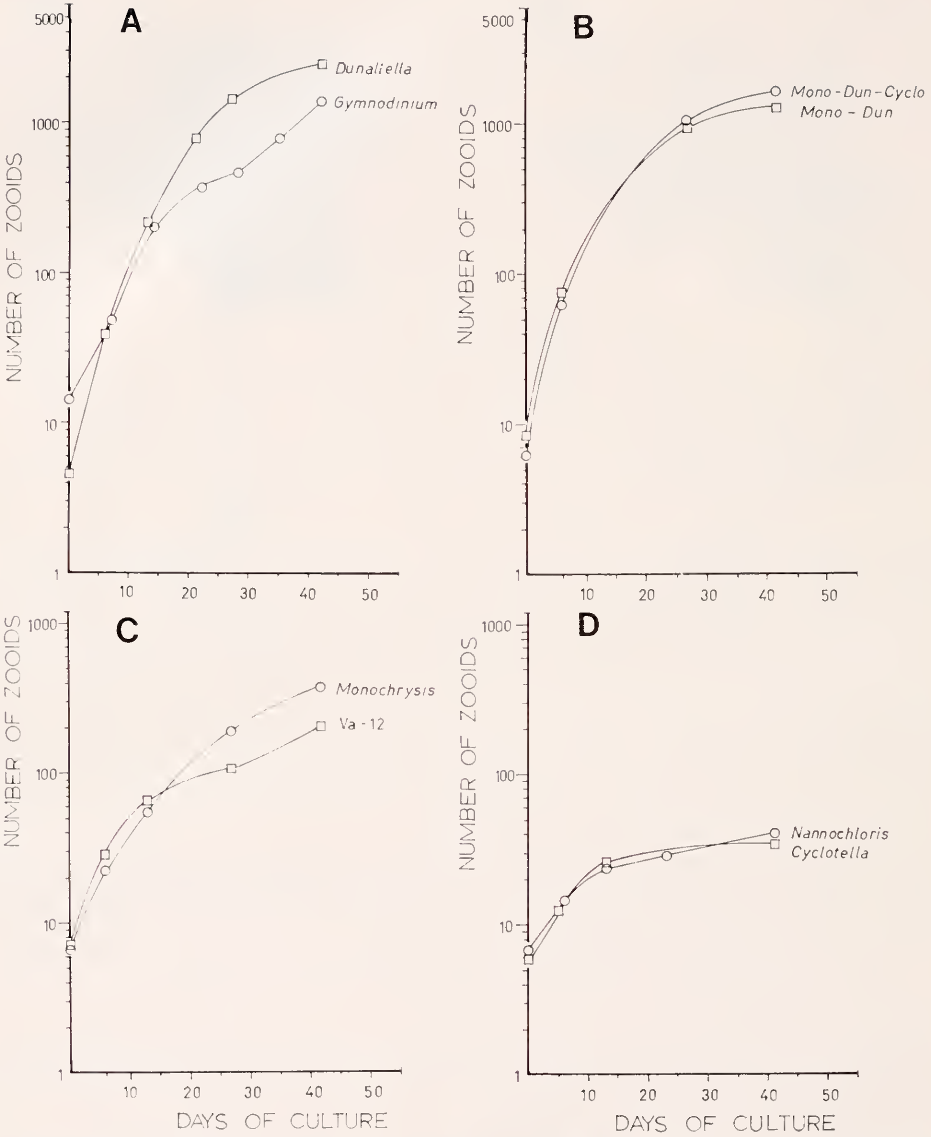

FIGURE 2. Mean growth curves for *Conopeum tenuissimum* colonies cultured on various algal foods. (A) Colonies raised on two good foods: *Dunaliella,* based on growth of seven colonies; *Gymnodinium,* based on growth of four colonies. (B) Colonies raised on algal food mixtures: *Monochrysis-Dunaliella-Cyclotella,* based on growth of six colonies; *Monochrysis-Dunaliella,* based on growth of eight colonies. (C) Colonies raised on two fair foods: *Monochrysis,* based on growth of five colonies, Va-12, based on growth of seven colonies. (D) Colonies raised on two poor foods: *Nannochloris,* based on growth of five colonies; *Cyclotella,* based on growth of eight colonies.

in the outermost zooids. In these colonies the gymnocyst appeared to be more heavily developed than in those fed on any other food and only inner portions of some colonies had any lateral spines (Fig. 1D).

Conopeum-fed *Anacystis marinus* (7/15–8/19; 35 days) grew poorly, but initial growth was greater than exhibited by colonies fed *Nannochloris* or *Cyclotella.* Unfortunately the stock culture of *Anacystis* was among the stock cultures affected by the VIMS culture failure (July 26, 1972), and it could not be regrown in time to start a new experiment. For the first eleven days of the culture period, the mean growth rate exceeded that of other poor-food cultures; but after the loss of the *Anacystis* culture, the growth rate was greatly retarded and lagged behind all other algal foods. There were, however, some live zooids present in all colonies at the end of the 35-day period.

Bacterial food (7/15–8/25; 41 days) appeared to be the worst of all foods tested for *Conopeum.* Growth was less than that exhibited by colonies given no food at all. At the end of the culture period, one colony had decayed completely. All other colonies had at least one functional polypide, but were obviously in a state of decay with many broken zooids.

Growth responses to food combinations

Conopeum was cultured on a bi-algal mixture of *Monochrysis* and *Dunaliella* (7/15–8/26; 42 days). This mixture proved to be a good food, giving better growth results than any single foods except *Dunaliella* and *Gymnodinium* (Fig. 2B). Post-culture examination showed one zooid with an ovary and several polypides with intertentacular organs. The shape of the colonies grown on this mixture was intermediate between that of colonies on a *Monochrysis*-diet and that of those on a *Dunaliella*-diet.

Conopeum colonies were also cultured on a tri-algal diet of *Monochrysis, Dunaliella,* and *Cyclotella* (7/15–8/26; 42 days). This tri-algal mixture also proved to be a good food. The mean growth rate was similar to the growth rate of colonies fed the *Monochrysis-Dunaliella* mixture, the only difference being that colonies given the three food combination had a slightly higher final mean zooid count (Table I). The tri-algal mixture also supported colony production of reproductive structures and an intermediate colony shape.

Growth of Conopeum *in the natural environment*

Attempts were made to assess growth of *Conopeum* in nature by placing colonies isolated on slides in a holder suspended from the VIMS pier into the York River. Unfortunately after about 20 days, overgrowth by other organisms was so intense that accurate zooid counts were impossible. A second attempt to assess natural growth was made by placing isolated colonies in an oyster culture tank receiving a continuous flow of York River water. This system eliminated the overgrowth problem. It was felt that this simulated natural conditions, except for lack of predators and a slightly lower water temperature. A comparison of the growth rate in the oyster tanks to cultured growth rates substantiated the belief that laboratory diets and cultures do serve to approximate growth in nature and can be used and interpreted in that light.

Control cultures

It was also deemed important to examine whether or not the algal medium itself could cause significant growth in *Conopeum* colonies. Colonies were cultured 41 days (7/15–8/25) on filtered sea water, using 60 ml of algal medium per feeding. Some initial colony growth occurred, but this was thought to be a result of polypide feeding on tiny diatoms which entered the culture *via* the glass slides used to isolate the colonies. At the conclusion of the culture, zooids of most colonies were broken or empty. The growth response was similar to that produced in colonies fed the diatom *Cyclotella*.

A second control experiment examined the growth potential of a colony given only its own food reserves and cultured in filtered sea water. Initial growth response produced a growth curve with a slope similar to those of *Cyclotella*, *Nannochloris*, and the algal medium. However, between two and three weeks after the start of the cultures the colonies started to decay. One colony had died by the 22nd day of culture. This colony had only two initial zooids at the start of

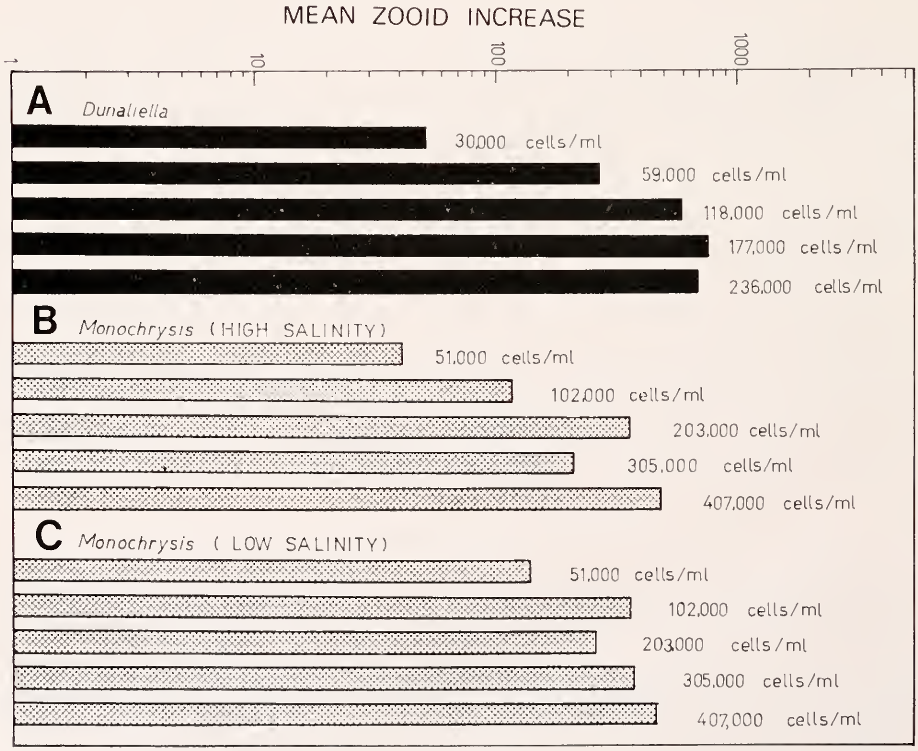

FIGURE 3. Growth of *Conopeum tenuissimum* colonies on various concentrations of algal foods (given as mean zooid increase): (A) *Dunaliella*, 15, 30, 60, 90, 120 ml per feeding; (B) *Monochrysis* (16‰ salinity), 15, 30, 60, 90, 120 ml per feeding; (C) *Monochrysis* (12‰ salinity), 15, 30, 60, 90, 120 ml per feeding. Figures indicate average concentration of the food (in cells/ml/l sea water) for each of the treatments.

culture and therefore a low nutrient reserve. However, another two-zooid colony survived 41 days, produced a total of six zooids and had one zooid with a polypide at the end of the culture. Of the other six colonies, four appeared to have at least one live zooid; the other two colonies were in a definite state of decay.

Growth response to various concentrations of food

Since food concentrations varied with both species and time, it was desired to test the effects of food concentration on colony growth. The results of three culture experiments utilizing similar concentrations of foods indicate that *Conopeum* growth was a direct function of food concentration up to about 60 ml. Above this amount there was no significant increase in growth. Figure 3 summarizes the results of the concentration of experiments.

The various concentrations of *Monochrysis* used to culture *Conopeum* generally supported moderate colony growth and a colony shape indicative of foods of moderate value (Fig. 1C).

Due to a change in laboratory water conditions, colony cultures using *Monochrysis* were carried out at two salinities. For the first series, salinity was 12‰ (7/17–8/26; 40 days).

Colonies fed various concentrations of *Monochrysis* at the low salinity supported growth, but there was frequent colony death. There were no colony deaths at the higher salinity, but there was evidence of zooid breakage. Other differences believed related to salinity were: (1) growth rates for the colonies fed small amounts of *Monochrysis* were lower for the colonies raised at the higher salinity than for those raised at the lower salinity; and (2) those colonies fed at lower salinities had more closely-grouped growth curves than those raised in the higher

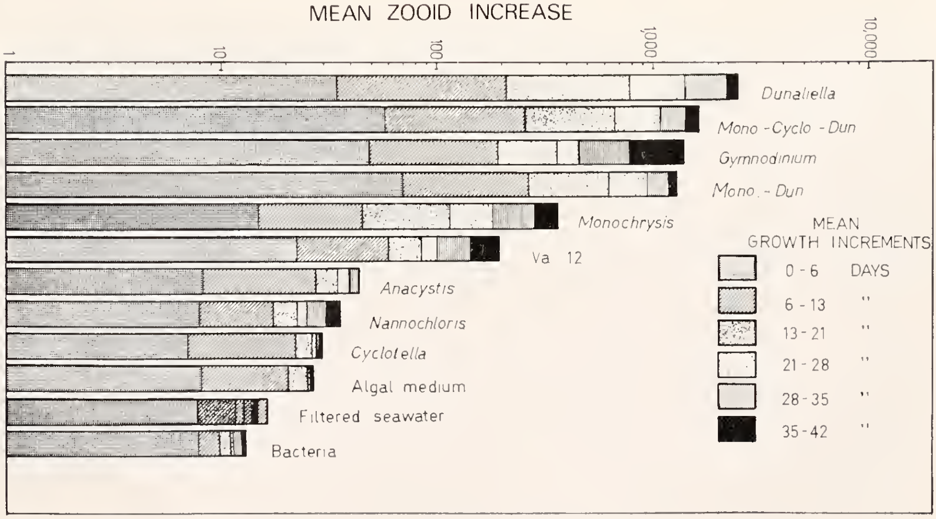

FIGURE 4. Growth of *Conopeum tenuissimum* colonies on all foods investigated (given as mean zooid increase).

salinity. The reasons for these differences are unknown but suggest further research.

Conopeum cultures on various concentrations of *Dunaliella* (8/21–10/2, 42 days) were compared to *Monochrysis*-cultured colonies. The *Dunaliella*-fed cultures had to be moved from VIMS to Woods Hole, Massachusetts (9/7/72), and the subsequent growth of these colonies reflected some degree of stress. The colonies did recover however.

From Figure 3 it is obvious that those colonies fed the smallest amount (15 ml) of *Dunaliella* did not grow appreciably more than those fed the smallest amount of *Monochrysis* at a similar salinity. Those fed 30 ml, however, showed slightly more growth than those fed 30 ml of *Monochrysis*. It is interesting that even in colonies fed the smaller amounts of *Dunaliella,* some showed the growth pattern characteristic of those on good diets, with zooids filling in between the main branches, rather than just growing outward in uniserial chains from the ancestrula.

The colonies fed 60, 90, and 120 ml of *Dunaliella* had very similar and closely spaced growth curves. Those fed 120 ml showed the least individual variation between individual colonies.

Colonies fed 60 90, and 120 ml of *Dunaliella* achieved better growth than those fed 60, 90, and 120 ml of *Monochrysis*. The *Dunaliella*-fed colonies showed quite circular growth patterns compared with those fed *Monochrysis*. When the colonies were examined at the end of the experimental period, none of the colonies showed any signs of reproduction except for the colonies fed 120 ml, which had polypides with intertentacular organs and ovaries in the zooids. It was observed that many of the polypides in the colonies fed 90 and 120 ml amounts of *Dunaliella* had lophophores that were shorter than normal and bodies that were like large sacks, possibly an adaptation to bloom conditions.

Discussion

To aid in the interpretation of results, bar graphs showing the amount of growth (as mean zooid increase) for *Conopeum* colonies on all diets were constructed (Figs. 3 and 4). For the food concentration experiments, Figure 3 shows only total mean zooid increase. The more detailed graph (Fig. 4) shows the amount of growth that occurred on each diet after 6, 13, 21, 27, 35, and 42 days. Growth at each of these points was measured directly from colony photographs when possible, and in a few cases interpolated from the mean growth curve. Examination of these figures and Figure 1 leads one to conclude that different species of algae affect both pattern and amount of colony growth.

The effect of food on colony growth patterns is of interest with respect to life strategy of this estuarine species. Colonies on a good diet (Fig. 1A) developed buds not only along the major growth axes, but also distally and laterally to fill in the spaces between the branches, giving the colony a circular rather than double-fan-like form. Colonies fed fair foods (*Monochrysis* and Va-12) generally grew in a biradiate pattern, in chains only one to two zooids wide (Fig. 1C). Though the chains leading out from the ancestrula in the distal and proximal directions were often the longest, in older colonies this was only on the order of a few generations of zooids compared with the length of the secondary chains (which generally developed from distal-lateral buds produced from the young colony, either before the experiment started or during the first week of growth). The secondary chains were usually at about a 45° angle (the angle at which distal-lateral buds are produced) to the primary chains; thus, colonies commonly consisted of six main chains or branches (three to either side of the ancestrula) in a bi-radiate pattern. Shorter tertiary chains developed by distal-lateral budding of the zooids of the primary and secondary chains.

This development in all directions outward from the young colony suggests that colony energy is being put toward the location of a more nutritionally favorable microenvironment. It is worth noting that colonies fed good foods did not have a larger number of generations (measured as number of zooids in a chain outward from the ancestrula). For colonies fed *Dunaliella* the mean number of generations was 33; for those fed *Gymnodinium*, 22; for the two and three-food mixtures, 29 and 36 respectively. For colonies given Va-12 it was 31; for those fed *Monochrysis*, it was 40. The poorly nourished colonies had mean generation numbers ranging from seven (bacterial-fed) to 15 (*Nannochloris*-fed).

What this means in terms of colony strategy can perhaps be roughly quantified by a simple ratio between final mean zooid number and final mean generation number (Table I). In well-nourished colonies, the amount of energy put into total colony growth including storage for maintenance and reproduction (as measured by final mean zooid number) versus the amount put into outward expansion (as measured by final mean generation number) ranges from 45:1 to 75:1, while ratios for those on fair foods ranged from 6.5:1 to 9.3:1. For colonies on poor diets, the range was from 2.3:1 (bacteria) to 4:1 (*Anacystis*). Thus colonies on a poor or fair diet were putting a proportionately much greater amount of energy into a "search" for a more favorable microhabitat, while well-nourished colonies were expanding as much as possible within their favorable habitat and were probably accumulating excess food energy (as shown by the onset of reproductive processes in these colonies).

What this could mean to colonies in their natural estuarine environment is apparent from a discussion of the life cycle (Dudley, 1973). If the colonies are not able to grow, store some energy and reproduce within about four weeks, colonies on hard substrates will become covered by other fouling organisms, such as colonies of the faster-growing, but less numerous ectoproct *Membranipora tenuis*, as well as by tunicates and barnacles. Their other primary substrate, the eel-grass, is short-lived in summer and may come detached and decay; therefore rapid completion of the life cycle is favored. Moreover, colonies in nature which find themselves in a nutrient-poor microenvironment due to current conditions probably also put all colony reserves into single chains of zooids, favoring expansion into an area where zooids can feed.

Examination of colony growth curves (Fig. 2) shows that the response does not differ significantly from the sigmoid growth curves characteristic of many animals (Odum, 1971). Growth curves of young colonies taken from the natural environment had an exponential form (Dudley, 1973). This type of curve also appeared in the early growth of cultured colonies. As the cultured colonies increased in size, the growth curves began to approach the sigmoid form. Exponential population growth can continue only as long as environmental factors are not limiting. As the number of zooids increase and factors such as space for colony expansion decrease, the growth rate slows until it reaches the carrying capacity of the environment. In the case of the cultured *Conopeum* colonies the chief constraint on growth was probably food supply. To simplify the experiments, colonies were fed a constant amount of food, and the food was not increased as the colonies grew; thus food supply might have been adequate for young colonies, but inadequate for the 1000–2000 zooids present in colonies at the end of the experimental period.

Other limiting factors may also be operating. It has been found true for many organisms that somatic growth diminishes or stops before sexual maturation, as energy is needed for the development of reproductive structures, eggs, brooding of larvae, etc. However, few studies are available, concerning the effects of the reproductive process upon the growth of colonial organisms.

As has been shown to be true for other organisms from protozoans to crustaceans, different species of algae were found to be of varying food value to *Conopeum* (see Fig. 4). Two single foods, *Dunaliella tertiolecta* and *Gymnodinium simplex,* supported good growth of *Conopeum.* The results with *Dunaliella* are interesting since species of this genus have been shown to be of quite variable value to other organisms (Walne, 1963, 1970; Gibor, 1956; Pilkington and Fretter, 1970). In addition Bullivant (1967) found no growth in *Zoobotryon* colonies fed *Dunaliella* except for an elongation of the stolons which seemed to be a reaction to adverse food conditions and Schneider (1959) stated that *Dunaliella* sp. passed intact through the gut of *Bugula avicularia.*

The results with *Gymnodinium* are perhaps even more interesting. Very few dinoflagellates have been used in culturing experiments, and the few species tried (which were generally species known to produce toxic metabolites) have usually given poor results. Buillivant (1967) found no growth and some disintegration in colonies of *Zoobotryon*-fed *Amphidinium carterae.*

Some dinoflagellates may have food value, as is suggested by the literature. Pilkington and Fretter (1970) reported that *Exuviella baltica* was a good food for *Crepidula* veligers. Moyse (1963) found that the small dinoflagellate *Prorocentrum micans* was able to support growth of the barnacle *Chthamalus stellatus* to the settlement stage.

It was probably significant that only the *Conopeum* colonies grown on *Gymnodinium* showed the natural reddish-brown coloration of the polypides, apparently due to the pigment of the dinoflagellate cells. One organism which has been cultured on dinoflagellates is a tintinnid (Gold, 1970). The author remarked that after overnight growth on the dinoflagellates *Glenodinium foliaceum* and *Amphidinium* spp., the tintinnids showed a characteristic coloration resulting from the ingestion of dinoflagellates. Since tintinnids are typical of estuarine zooplankton it is not so surprising that they, like the estuarine ectoproct *Conopeum,* appear to thrive on dinoflagellates (also common in estuaries).

Gymnodinium simplex had been chosen as a food organism because it was known to occur in the York River and could thus be considered a "natural" food organism for *Conopeum.* Furthermore, information available on the dinoflagellates of the York River (data in MacKiernan, 1968) indicated that about 50% were of a size (diameter 50 μm) that would make them available as food for *Conopeum.* In the warm temperate York River estuarine environment, dinoflagellates occurred year-round and some suitably-sized species were always present. In addition, various nannoflagellates were common especially in summer months, while diatoms did not appear to be significant as food for *Conopeum* because most of the species reported were too large for the ectoproct to ingest. Thus it appears that the value of *Gymnodinium* as food for *Conopeum* might actually reflect the natural food of this organism. Whether this finding can be extrapolated to other estuarine ectoprocts remains to be seen.

The two foods found to support fair growth in *Conopeum* were *Monochrysis lutheri* and Va-12. On those diets, the colonies were able to grow moderately well, but did not achieve reproductive conditions by the end of the experimental period. Though both species were chrysophyte flagellates of similar size and volume, *Monochrysis* proved to be a better food than Va-12.

Monochrysis has been used in many culture experiments and has been shown to be one of the best foods for oyster larvae (Davis and Guillard, 1958; Walne, 1963). Va-12 has been used only in the VIMS larval culture work where it was found to increase the growth rate of oyster larvae when mixed with other foods, but was unable to support growth in monoculture (M. Bolus, VIMS, unpublished manuscript).

Several foods supported only poor growth of *Conopeum* colonies. The cyanophyte *Anacystis marinus* was the best of the poorer foods and might support moderate growth. Schindler (1971) pointed out the ingestion and assimilation of blue-green algae indicated that no generalization could be made about the food value of those algae on taxonomic grounds, although complex or filamentous forms were unsuitable. His experiments suggested that members of this group could be potential food sources at least for freshwater suspension feeders. No attempts appear to have been made to culture estuarine organisms on blue-green algae, but as members of this group can be of considerable importance, particularly in the upper reaches of estuaries, their food value to suspension-feeders should be more thoroughly investigated.

The very small chlorophyte *Nannochloris occulata* was a poor food for *Conopeum*. This may have been partly because of its size (perhaps the cells pass between the cilia and cannot be captured by the lophophore) although the *Anacystis*, with cells in the same size range, was apparently more efficiently utilized.

The small centric diatom *Cyclotella nana* (= *Thallassiosira pseudonana*) also appeared to be of little food value to *Conopeum*. Bullivant (1967) found that *Cyclotella* produced some growth of *Zoobotryon*, a gizzard bearing ctenostome, but not of *Bugula*, a species lacking a gizzard. I have also found that *Cyclotella* does not support growth of *Bugula stolonifera*.

The colonies cultured on bacteria showed even less growth than those receiving only filtered sea water. While there has been much speculation in the literature concerning the role of bacteria in the nutrition of suspension-feeding organisms, most attempts to culture invertebrates on bacteria have ended in failure. Bacteria in combination with phytoplankton may play some role in bivalve nutrition, but it is still unclear whether they are generally useful or harmful (Ukeles, 1971). It appears that certain species of bacteria produce toxic metabolites and others do not (Calabrese and Davis, 1970). The bacteria utilized in the *Conopeum* experiments could not have produced extremely toxic products as the colonies did not die immediately. The reason for poor growth may be one or a combination of the following: the cells were present in too low a concentration to have nutritional value; or, as has been shown to be the case for many marine bacteria (Provasoli, Conklin and D'Agostino, 1970), they created an unfavorable environment for the ectoprocts by acidifying the sea water and reducing the oxygen concentration. The nutritive value of algae appears important in that not all foods that supported growth were adequate for reproductive activity. The fact that not all the foods tested were able to support reproductive activity of *Conopeum* may be accounted for either by assuming that: (1) not all algae are alike in food value, especially in the amounts of trace elements, vitamins or other factors necessary for reproduction; or (2) colonies cannot undergo reproduction until they reach a certain size, as measured in the number of zooids. Actually both factors may be operating, but in the absence of any knowledge of a minimum-size requirement (and such a requirement would seem to be less likely in an r-strategist like *Conopeum,* than in a generally long-lived species which puts its energies first into obtaining as much substrate as possible by asexual growth), it is most probable that the food is lacking in some element necessary for the initiation of the reproductive processes.

The survival of some colonies for as long as six weeks, during which time they received only filtered sea water, suggests that colony reserves are important in insuring the survival of the species in the event of unfavorable conditions. As is apparent from Figure 4, growth of the colonies fed good, fair and poor foods is distinguishable even after only six days of culture. The control colonies (filtered sea water) grew as much as the colonies fed poor foods for the first six days. Between six and thirteen days the controls showed more growth than the colonies fed bacteria, but were apparently becoming more and more unhealthy. By the time they were photographed again at 21 days, zooids had degenerated and decayed so that the total mean zooid number was less than it had been after only 13 days. From 21 days to 42 days the colonies made only a tiny increase in growth (from $\bar{x} = 12$ to $\bar{x} = 15$ zooids/colony). This very small increase may have been due to remaining food reserves or to ingestion of benthic diatoms or bacteria that could not be completely removed from the dishes or the surfaces of the colonies.

For colonies receiving 60 ml of sterilized algal medium per feeding, growth continued up until the third week (Fig. 4), and even after six weeks was almost equivalent to the growth of colonies receiving *Cyclotella.* As was mentioned in the results section, continued growth may have been due to the fact that numbers of small benthic diatoms were encouraged by the algal medium and might provide some nutrition for the *Conopeum* colonies.

Conopeum growth response to a combination of algal foods was better than to most single foods. Colonies fed a mixture of two foods, *Monochrysis* and *Dunaliella,* showed better growth than colonies fed all but two single foods (Fig. 4). Moreover, the growth curves for all of the colonies grown on the two-food mixture were remarkably similar. The results are not surprising since a mixture of foods has been found to support better growth than a single algal food for many other suspension feeders (Davis and Guillard, 1958; Ukeles, 1971; Provasoli, Conklin and D'Agostino, 1970).

Growth of *Conopeum* on a mixture of three foods (*Monochrysis-Dunaliella-Cyclotella*) was slightly better than on a mixture of two foods and better than on any single food except *Dunaliella*. Calabrese and Davis (1970) stated that a mixture of several species of algae caused larvae of *Crassostrea* and *Venus* to grow more rapidly. They found that a mixture of chrysomonads and green algae (no species given) appeared to provide a more balanced diet than any single food. The fact that, in a 2–3 food mixture, the *Conopeum* colonies were receiving only one-half or one-third the amount of the "good" food suggests that the important factor lies in some form of nutrient balance rather than in the quantity of nutrient offered. The culture experiments using various concentrations of algae showed that food value increased with increasing amounts of food only up to a certain concentration. Figure 3 shows the effect of increasing food concentrations on colonies fed *Dunaliella* and *Monochrysis* (at high and low salinities). In the colonies fed *Dunaliella* (Fig. 3A), growth increased at food concentrations up to 177,000 cells/ml (average quantity), but seemed to be slightly decreased in colonies receiving an average of 236,000 cells/ml. This decrease could be accounted for by two factors. First, as several workers have noted, suspension feeders will ingest food in increasing quantities only up to a certain "satiation concentration" related to the maximum amount of food the individual can process in a certain time period (Bullivant, 1967). If the highest concentration tested was above the maximum that the colonies could consume during the time between feedings, then there could be no increase in growth due to increased concentration. Secondly, it is also possible that *Dunaliella* produces a metabolite that at high concentrations has a negative effect on growth (which might explain the varied results noticed by other workers in culturing experiments with members of this genus).

Colonies grown on *Monochrysis* in general showed an increase in size with increasing amounts of nutrient, but there was more irregularity in the pattern. Colonies grown at the lower salinity (Fig. 3C) appeared to grow better at the two lower levels of food than did those grown at the higher salinity (Fig. 3B). At the highest concentration (406,800 cells/ml) offered, both series achieved approximately equal growth.

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SUMMARY

1. The pattern and form of colony growth in the ectoproct *Conopeum tenuissimum* was found to be diet-dependent.

2. Poorly nourished colonies were characterized by their straggling shape and low zooid number to generation number ratios. These colonies apparently attempted to maximize substrate covered, facilitating location of a more favorable nutrient regime.

3. Well fed colonies were rounded in shape and characterized by high zooid number to generation number ratios. These colonies maximized the number of zooids produced in their already favorable area, creating colony reserves, and preparing for reproduction.

4. Initial growth in all colonies was exponential, but after a few days of culture growth, rates varied with food used. The chlorophyte flagellate *Dunaliella tertiolecta* and the dinoflagellate *Gymnodinium simplex* were good foods, supporting growth to more than 1,000 zooids and sexual maturation within the 42 day culture period. The chrysophyte flagellate *Monochrysis lutheri* and Va-12 (an un-named chrysophyte flagellate) proved to be fair foods, supporting moderate growth, but no sexual maturation. The blue-green alga *Anacystis marinus*, the chlorophyte *Nannochloris occulata*, and the diatom *Cyclotella nana* supported little or no growth, while bacterial food did not support growth of *Conopeum* cultures.

5. Combinations of algal food produced good colony growth. A three-species mixture was better than a two-species mixture, suggesting differential yet additive nutritional contributions by each algal species.

6. Increased food concentrations supported increased growth only up to a certain concentration. This growth response varied with salinity.

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WINSTON, J. E. See Dudley, J.

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3. ***A condensed title*** or running head of no more than 35 letters and spaces should be included.

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ERRATUM

THE BIOLOGICAL BULLETIN, Volume **150**, Number 1, Pages 144 and 145

The following correction should be made in the paper by Michael A. K. Smith and Alan Thorpe entitled, Nitrogen metabolism and trophic input in relation to growth in freshwater and saltwater *Salmo gairdneri* (1976, *Biol. Bull.,* **150**: 139–151):

On pages 144 and 145 the line-graphs are reversed. The graph with the diverging lines should be Figure 2. The captions and all other references are correct as printed.

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THE

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THE ELECTROPHYSIOLOGY OF THE ORGAN-PIPE CORAL, *TUBIPORA MUSICA*

Reference: *Biol. Bull.*, **150**: 337–347. (June, 1976)

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Although corals constitute remarkable examples of colonial organization, their behavior has received little attention since Horridge studied the effects of mechanical and electrical stimulation on a variety of corals (Horridge, 1956, 1957). He found two separate yet sometimes interacting levels of behavior: that of the individual polyps and that of the colony as a whole, with the latter involving coordinated activities of two or more polyps.

Individual polyps were found to feed, withdraw and perform other activities without affecting the remainder of the colony. However, with appropriate stimulation, this autonomy broke down and colonial responses involving retraction of large numbers of polyps were evoked. Such colonial responses spread in one of two ways. Certain corals, typified by *Tubipora*, responded in a through-conducting manner, with each wave of activity affecting all parts of the colony. In other corals, the spread was incremental, each stimulus serving to increase the size of the responding area (Horridge, 1957).

Apart from the question of how incremental spread can occur in a conduction system composed of all-or-nothing conducting units, such behavior poses two problems. First, how can the behavior of individual polyps be kept separate from those of the remainder of the colony; and second, how can that autonomy be broken down so as to produce a colonial response?

In the pennatulid *Renilla köllikeri*, the autonomy of individual polyps is maintained by the necessity for a complex and not fully understood interaction between two conduction systems within the colony (Anderson and Case, 1975). One conduction system, the Zooid Nerve Net (ZNN) is restricted to individual polyps where it is responsible for polyp withdrawal. The ZNN can be activated by a through-conducting colonial nerve net. While colonial nerve net activity can simultaneously activate the ZNN in all the polyps, there is a degree of flexibility inherent

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in the interaction that allows each autozooid to retain a measure of control over whether or not it withdraws during colonial nerve net activity.

Through-conducting nerve net activity has been recorded from a variety of alcyonarians (Shelton, 1975c; Anderson and Case, 1975) and more recently from certain madreporian hard corals (Shelton, 1975a, d). Furthermore, there is good evidence that there are two colonial conduction systems in certain pennatulids (Shelton, 1975c; Anderson and Case, 1975) and perhaps some hard corals (Shelton, 1975d). However, with the exception of *Renilla,* no colonial anthozoans have been studied from the viewpoint of how the autonomy of the individual polyps is maintained.

Horridge's descriptions of the behavior of *Tubipora* (Horridge, 1957) indicate differences between it and *Renilla* in the mechanisms by which colonial activity affects the polyps. In *Renilla* there are varying requirements for the interaction between the colonial conduction system responsible for colonial polyp withdrawal and the conduction system in each polyp. Different polyps respond to different numbers of colonial pulses. The result is that colonial polyp retraction is spatially irregular; widely separated polyps may contract nearly synchronously, while there may be major delays between the onset of contraction in neighboring polyps. In *Tubipora,* polyps withdraw in sequence so as to produce a wave of twitching and withdrawal that spreads across the colony away from the point of stimulation.

This rather large difference in the behavior of the two colonies suggests that the mechanisms by which the autonomy of the polyps in *Tubipora* colonies is maintained may differ from that described for *Renilla.* Consequently, this problem was given special attention during this study which comprised part of a series of investigations of conduction systems in colonial anthozoans.

Materials and Methods

Specimens of *Tubipora musica* Linnaeus (sub-class Alcyonaria, order Stolonifera) were collected from the reefs surrounding the Banda Islands, Indonesia. Large populations of the coral were found only in a few sheltered, shallow locations. Large colonies, frequently measuring up to three feet in diameter were observed underwater, while more detailed studies were conducted on 8–12 cm diameter colonies maintained in aquaria. At all stages of the collections and experiments, great care was taken to ensure that the colonies were never removed from the water. When colonies were exposed to air, bubbles became trapped between the pipes that comprise the colony and remained there upon submersion. This resulted in rapid decay of the colony. The water in the aquaria was changed regularly and filtered continually. The colonies were not fed. It did not prove necessary to control the temperature of the water in the aquaria since it maintained itself at approximately 28° C, the temperature of the surface water in the region. Electrical recordings were made with suction electrodes in the manner described previously (Anderson and Case, 1975).

Results

Tubipora musica colonies are composed of parallel arrays of pipes or tubes linked together at regular intervals along their length by connecting platforms

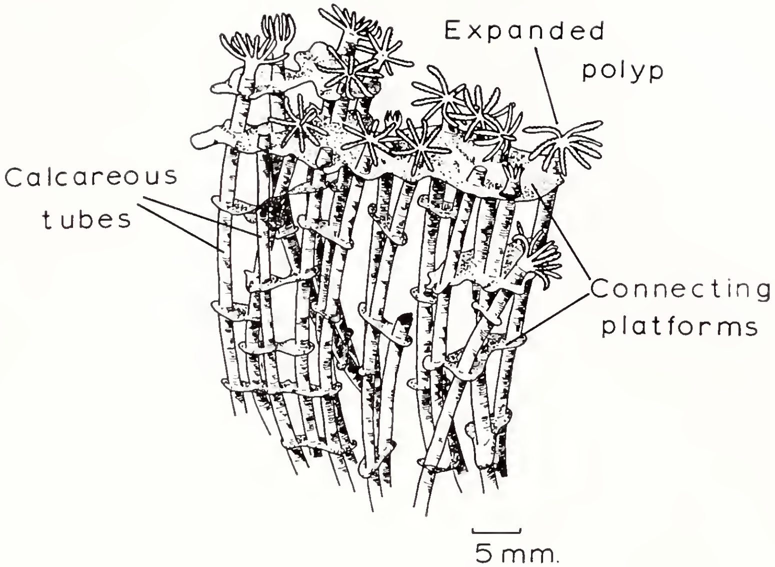

FIGURE 1. Diagram of a small portion of a *Tubipora musica* colony.

(Fig. 1). The skeletons are a dull red color but the living colonies usually appear various pale shades of green, blue or purple due to the color of the expanded polyps. Generally any one colony is composed of polyps of a single color, although occasionally portions of large colonies were found to be composed of polyps of a different color from that of the remainder of the colony. In many of these cases, there is a distinct boundary between the two color regions. If one part of such a coral was stimulated, the resulting wave of withdrawal would pass across the boundary, indicating that these specimens were functionally single colonies.

Since the behavior of *Tubipora* has been well described by Horridge (1957) only a summary is given here with a few additional observations. When a tentacle of a polyp was lightly touched with a probe, it, and the neighboring tentacles of the polyp, shortened and bent away from the oral disk. Only rarely would a tentacle bend toward the oral disk in the manner described by Horridge. If the same tentacle were touched again, all the remaining tentacles shortened and bent away from the mouth; and a third such stimulus caused polyp withdrawal. During withdrawal the tentacles were elevated and the polyp pulled down into its tube in discrete twitches. Withdrawal of one polyp usually had no effect on the remainder of the colony. If stimuli of higher intensities than those required to evoke responses from individual polyps were used, a colonial response was invariably produced. In *Tubipora* there appeared to be only one colonial response; polyp retraction. A mechanical stimulus evoked a wave of polyp retraction which spread across the colony away from the point of stimulation. It proved very difficult to control the intensity of the mechanical stimuli to the degree that only one wave of polyp retraction was produced, and frequently, several successive waves of twitching followed each stimulus. During the colonial response, the withdrawal behavior of the polyps appeared almost identical to that described above for withdrawal of polyps stimulated individually. That is, the first wave of colonial activity involved a shortening and a downward movement of the tentacles of the polyps, while the second wave consisted of an upward flexion of the tentacles prior to actual retraction of the polyp, an action which was completed in discrete stages during the succeeding waves of colonial activity.

Similar colonial responses could be evoked with electrical stimulation. A single supra-threshold stimulus did not produce retraction but apparently activated the colonial conduction system, as evidenced by scattered tentacle waving of polyps across the colony. A second stimulus produced a single twitch from all the polyps and as described above, this involved shortening and depression of the tentacles. Successive stimuli evoked further waves of activity with each of the above-mentioned stages in the retraction response comprising the activity associated with each wave. The size of the twitches or movements associated with each stage of the response was greater at shorter interstimulus intervals.

Electrical activity associated with this behavior was recorded with suction electrodes attached to the colony. While potentials could be recorded from all parts of the polyps, clear, long-term records could only be obtained from the oral disk. Electrical stimuli were applied through an additional suction electrode, frequently attached to a polyp in a part of the colony displaced from the recording sites. Each

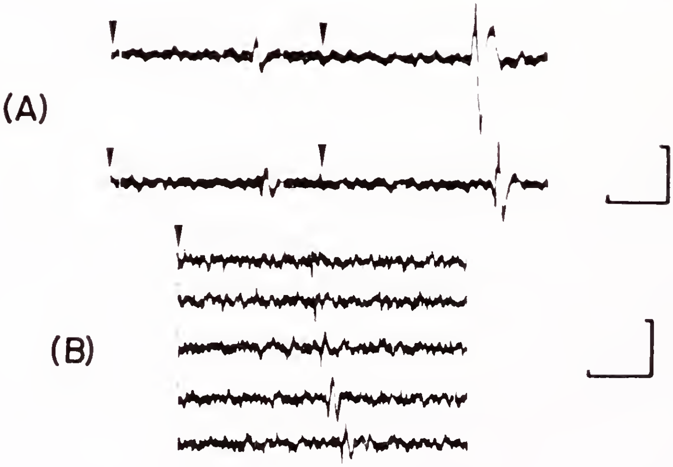

FIGURE 2. (A) Electrical activity recorded from *Tubipora* through two suction electrodes attached to different polyps. Each of two electrical stimuli (marked with a dark triangle) evokes a single through-conducted potential. While the potential evoked by the first stimulus consists of a single unit, the potential evoked by the second stimulus contains an additional component interpreted as a muscle action potential. (B) Facilitating potentials recorded through a single electrode. Five stimuli were applied at two second intervals. The first stimulus gives rise to a single small potential that reappears after all subsequent stimuli. Later stimuli evoke potentials composed of an additional facilitating muscle action potential. The ordinate is 10 μV, abscissa, 200 msec.

electrical stimulus evoked a single through-conducted potential (Fig. 2A). The potential evoked by a single stimulus or the first of a series was a simple, invariant, small amplitude (5 μV), short duration (20 msec) impulse that was never associated with any colonial activity other than the scattered tentacle waving mentioned above. Subsequent stimuli evoked more complex potentials composed of two parts: an initial component identical to that evoked by the first stimulus of the series, and a second, slower and larger component. Both the amplitude and the duration of the second component varied with the interstimulus interval, being greatest at shorter intervals. [Compare Fig. 2A (interstimulus interval approximately 650 msec) with Fig. 2B (interstimulus interval, 2000 msec).] During a series of pulses, the amplitude and duration of the second component increased and later decreased, the size at any one time being a function of stimulus frequency and pulse number. The presence of the two components is apparent in most but not all records.

Occasionally, the second component would be recorded after a single stimulus. Whenever this happened, however, there was a distinct colonial response, beyond the simple tentacle waving normally associated with a single stimulus. Since the second component was always found to be associated with polyp retraction, it was interpreted as being a muscle action potential associated with activation of those muscles involved in polyp retraction. As the interval between a pair of stimuli was increased, the amplitude of the muscle action potential evoked by the second shock declined. In some trials, clear muscle action potentials followed shocks separated by as much as ten seconds, but the neuromuscular facilitatory period was quite variable and often considerably shorter than ten seconds. In behavioral experiments in which the interval between two stimuli was increased until the second had no apparent effect, Horridge (1957) found that the facilitatory period for *Tubipora* was about four seconds.

Mechanical stimuli strong enough to evoke a colonial response evoked potentials identical to those that follow electrical shocks. Each mechanical stimulus usually evoked a single potential. While two potentials sometimes followed a single mechanical stimulus, bursts of more than two were never recorded. When mechanical stimuli of the strength necessary to produce multiple waves of twitching were used, all records subsequent to the application of the stimulus were obscured by a movement artifact. Horridge (1957) found that a strong mechanical stimulus gave rise to successive waves of twitching which spread across the colony at a rate of 2/second. In this study the mean inter-pulse interval for the paired potentials recorded after some mechanical stimuli was 541 msec, a figure that corresponds well with that found by Horridge (1957).

The conduction velocity of the through-conducted potentials was determined from the difference in the response latency between two recording sites. The conduction velocity was greatest for the first potential in a series (mean value 18 cm/sec at 28° C) and diminished for subsequent potentials. The final value of the conduction velocity during repetitive stimulation depended on the frequency and number of stimuli. For example, in one trial, the conduction velocity fell from 18 cm/sec after the first stimulus to 12 cm/sec after the fifth stimulus of a series applied at one per second. The relative refractory period was 250 msec.

Potentials similar in shape to those evoked by stimulation were recorded spontaneously at a rate of approximately one to two every minute. Some of these were through-conducted, appearing almost simultaneously on the records from widely separated polyps. Others were not through-conducted, but appeared only on records from a single polyp. For clarity, the former class of spontaneous potentials will be referred to as the spontaneous through-conducted potentials, and the latter as the isolated potentials. Neither type of spontaneous potential appeared to be recorded in any obvious pattern, and there were no apparent interactions between them.

Isolated potentials were restricted to the polyp in which they originated. If two electrodes were attached to adjacent polyps, spontaneous isolated potentials were still recorded, indicating that these potentials were not propagated between neighbors. In further tests to verify that there was indeed no propagation of isolated potentials out of individual polyps, two recording electrodes were attached to widely separate polyps. The polyps surrounding one of those bearing an electrode were then lightly touched and made to retract. In almost every case, the stimulated polyps could be made to retract completely without potentials being recorded by the electrode on the adjacent polyp. In the few cases when a potential was recorded from the adjacent polyp the simultaneous appearance of a similar potential on the record from the more distant electrode revealed that the recorded potential was a result of activation of the colonial conduction system. If the polyp to which an electrode was attached were touched lightly until a tentacle shortened or bent down from the oral disk, an isolated potential was recorded.

To test whether the observed spontaneous potentials were the same as the stimulated ones and not potentials associated with other unidentified conduction systems, electrical stimuli were applied, in the usual manner, immediately after a spontaneous potential was observed. When this was done after a spontaneous through-conducted potential had been recorded, a single through-conducted potential was recorded from both electrodes. The delay between the stimulus artifact and the recording of the potentials on each trace matched that which would be expected for a stimulated through-conducted potential traveling through the pathways involved. However, the stimulated potentials invariably included muscle action potentials and were therefore larger than those normally evoked by a single stimulus. The presence of the muscle action potential on the stimulated potential indicated that the spontaneous potential must have had the same effect on the muscles of a polyp as a stimulated potential, and therefore proved that both types of through-conducted potentials were identical. The appearance of the spontaneous potentials in the apparent absence of stimulation may have been a consequence of mechanical stress caused by the presence of the electrodes. They may also be a feature of undisturbed colonies, but no evidence was available to support this possibility.

Similar experiments were done to ascertain whether the isolated potentials were the same as the through-conducted ones. These experiments were preceded by a series of controls. These controls consisted of a pair of electrical stimuli applied to the colony, in the usual manner, at the beginning of an experiment, and at random intervals during it. Normally, the potentials evoked by stimulation of the

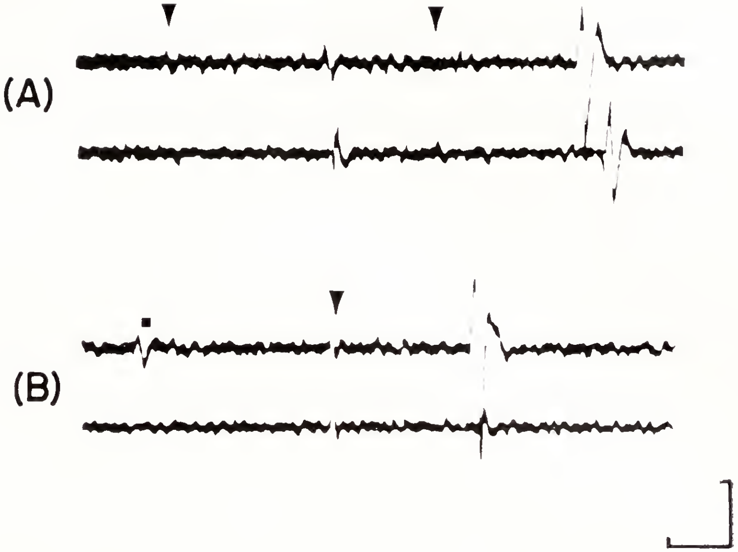

FIGURE 3. (A) Electrical activity recorded as in Figure 2A. These records served as a control for later experiments illustrated in Figure 3B. (B) Effect of electrical stimulation of *Tubipora* after an isolated potential (marked with a dot on the upper trace) has been recorded. Note that the amplitude of the potentials evoked by the electrical stimuli are no longer equivalent. (Ordinate and abscissa are as for Figure 2.)

colonial conduction system were equivalent. That is, after a single stimulus a single, small potential was recorded from polyps all over the colony; while a second stimulus evoked potentials which contained both muscle action potentials and the small impulse triggered by the first stimulus (Fig. 3A). Invariably, the muscle action potentials are of equivalent sizes. When a single stimulus was applied after an isolated potential had been observed, two through-conducted potentials were recorded (Fig. 3B). Unlike the potentials recorded after the control stimuli, these potentials were not of an equivalent size. In Figure 3B, the evoked potential on the lower trace consists only of the small potential typical of that normally evoked by the first stimulus of a series. The evoked potential on the trace containing the isolated potential (upper trace) contains a muscle action potential. In other experiments, both evoked potentials occasionally contained a muscle action component. However, in these instances, the muscle action potential recorded from the same polyp as the isolated potential was always larger. This evidence indicates that a single, isolated potential must have had the same effect on the muscles of a polyp as a single through-conducted potential; it facilitated those muscles so that a second potential, whether through-conducted or not, could activate those muscles and produce a twitch of the polyp.

The effect of magnesium ions on the colonial conduction system was determined by replacing the sea water in the preparation bath with a 50% solution of isosmotic $MgCl_2$ in sea water. Recordings were made during the perfusion process, and the colony stimulated once every two minutes with a train of three or four stimuli. Normal facilitating potentials were recorded after 55 minutes of immersion in the magnesium sea water.

DISCUSSION

Polyp retraction is the only observable colonial behavior of *Tubipora musica*. While it is possible that others do exist, they have not been revealed by either this study or that of Horridge (1957). The conduction system responsible for control of this behavior is through-conducting over the entire colony. Potentials recorded from this conduction system are composed of two components: a small potential that can be recorded from the conduction system itself; and a second, larger, facilitating potential that has been interpreted as a muscle action potential. It is probable that the muscle action potentials originate in the muscles responsible for polyp retraction. Because of the relationship between the triggering potential from the colonial conduction system and the muscle action potentials, the recordings from *Tubipora* resemble many of the classic facilitating neuromuscular potentials reported by previous investigators (Josephson, 1966; Robson and Josephson, 1969; Pickens, 1974).

Perfusion of the preparation bath with magnesium sea water had little effect on both the propagation of potentials through the colony and the appearance of the facilitating potentials recorded from the polyps. It is generally accepted that whereas excess Mg^{++} ions interfere with nervous and neuromuscular processes, they have little effect on epithelial conduction systems. This conclusion has been derived from the effects of solutions similar to those used here, on siphonophores (Mackie, 1965) and hydromedusae (Mackie and Passano, 1968) where both epithelial and neural conduction systems have been identified by histological, physiological and behavioral criteria. The effect of magnesium ions alone is far from sufficient evidence for claiming that a conduction system is neuroid. However, the degree of insensitivity to magnesium displayed by *Tubipora* is surprising, particularly when considered in terms of the effect of magnesium on other anthozoans. Excess Mg^{++} rapidly blocks activity in all three identified conduction systems in sea anemones (Robson and Josephson, 1969; McFarlane, 1969), in the two colonial conduction systems in the pennatulid *Renilla köllikeri* (Anderson and Case, 1975) and completely anesthetizes colonies of the madreporarian hard coral *Goniopora lobata* (Anderson, in preparation). Certain of these conduction systems, the SS1 and SS2 in anemones (McFarlane, 1969; Shelton, 1975b) and one of the colonial conduction systems in pennatulids (Shelton, 1975c) may be epithelial. The action of magnesium in all these cases is rapid, in complete contrast to the results found for *Tubipora*. It is conceivable, however, that the insensitivity displayed by *Tubipora* may be a result of inaccessibility of the nerves or some other factors. Consequently, it is too early to designate a morphological basis to the colonial conduction system responsible for polyp retraction. In the meantime, it will be referred to simply as the colonial conduction system.

All potentials recorded in this study have been shown to have originated in a single conduction system, the colonial conduction system. The absence of multiple conduction systems may be somewhat atypical of anthozoans. In sea anemones, there are, in addition to the through-conducting nerve net, two "slow conduction systems" (McFarlane, 1969). In some pennatulids, there are two colonial conduction systems (Shelton, 1975c; Anderson and Case, 1975), a through-conducting nerve net and a second system which has been equated with the "slow systems" in anemones (Shelton, 1975c). There is also circumstantial evidence of a "slow conduction system" in certain hard corals (Shelton, 1975d). While it is conceivable that improvements in recording techniques may reveal potentials originating in other conduction systems, the apparent absence of multiple conduction systems in *Tubipora* may be a reflection of a limited repertoire of colonial behavior.

A mechanism by which the autonomy of individual polyps can be maintained emerges from these results. Isolated potentials are restricted to individual polyps where they appear to originate in a conduction system responsible for controlling the behavior of the polyp. This is evidenced by the fact that mechanical stimulation of a polyp evokes an isolated potential. Similar stimuli can also provoke withdrawal of a polyp through a series of stages very reminiscent of the same process in sea anemones (Pantin, 1935 a,b,c,d). Because the isolated potentials are identical to through-conducted potentials, both in their shape and their effect on the muscles of a polyp, it would appear that the potentials recorded during colonial activity are in fact isolated potentials that are evoked sequentially in all the polyps by the wave of colonial activity that spreads away from the stimulating site. It is not possible to say at this stage, whether the conduction system in the polyps is an integral part of the colonial conduction system or a second conduction system which is triggered once by each colonially conducted potential. It is apparent, however, that the connection between the conduction system in the colony and that in the polyp is not through-conducting in both directions. Through-conducted potentials can enter polyps easily. This is illustrated by the fact that each stimulus applied to the colonial conduction system evokes a potential which can be recorded from polyps all over the colony. However, potentials which originate in a polyp, isolated potentials, rarely pass into the colonial conduction system and only then in instances when a polyp has been strongly stimulated. This would indicate that there is some kind of polarization of the connection between the conduction system in individual polyps and that operating through the colony as a whole. Since the morphological basis of the conduction system in *Tubipora* is unclear, speculation as to the means by which the polarization could be brought about would be pointless. Polarization of a similar nature has been described in the colonial hydroid *Hydractinia* (Stokes, 1974) where stimulation of the basal mat can evoke a propagated response involving lashing of dactylozooids. Direct stimulation of a dactylozooid does not, however, affect other dactylozooids in the colony.

The arrangement of conduction systems in *Tubipora* is similar to that already described for *Renilla* (Anderson and Case, 1975) with the exception that there is apparently only one colonial conduction system in *Tubipora*. The colonial conduction system responsible for controlling polyp retraction is through-conducting in both cases, and polyp withdrawal may involve activation of a second conduction system restricted to individual polyps. However, the arrangement in *Tubipora* differs in that each colonially conducted potential affects the muscles of the polyps, with facilitating muscle action potentials being recorded after every stimulus except the first of a series. In *Renilla* several colonially conducted Nerve Net pulses are required before a burst of Zooid Nerve Net pulses is produced and withdrawal occurs. Because *Tubipora* polyps respond to every colonial potential, flexibility inherent in the interaction between the colonial Nerve Net and the Zooid Nerve Net in *Renilla* polyps is lost. Despite this, *Tubipora* achieves the same end of having a mechanism whereby the activities of any one polyp can be kept separate from others in the colony. However, this separation is not so complete that a colonial response cannot occur.

I am very grateful to Dr. J. F. Case for allowing me to participate on the expedition and for his comments on the manuscript. My thanks also to Dr. R. K. Josephson for helpful discussions during the course of the experiments and his valuable comments on the manuscript. This paper is part of a thesis to be presented to the Department of Biological Sciences, University of California, Santa Barbara in partial fulfillment of the requirements for the Ph.D.

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SUMMARY

1. Through-conducted electrical activity can be recorded from colonies of *Tubipora musica* after mechanical and electrical stimulation.

2. The recorded potentials, conducted with a maximum velocity of 18 cm/sec, show distinct facilitation of a component interpreted as a muscle action potential. The colonially conducted potentials responsible for muscle activation in the polyps appear on the records as small (5 μV) potentials that precede the muscle action potentials.

3. The propagation and form of all recorded electrical activity is unaltered after 55 minutes submersion of *Tubipora* in a solution containing high magnesium.

4. Commonly, potentials similar to those associated with colonial activity can be recorded from only one polyp. These are interpreted as being the result of some form of polarization between the conduction system in the polyp and that in the colony which allows individual polyps to function independently of the remainder in the colony.

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LARVAL DEVELOPMENT OF THE NORTHERN HORSE MUSSEL, *MODIOLUS MODIOLUS* (L.), INCLUDING A COMPARISON WITH THE LARVAE OF *MYTILUS EDULIS* L. AS AN AID IN PLANKTONIC IDENTIFICATION[1]

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The northern horse mussel, *Modiolus modiolus* (L.), is found along the coasts of North America from the Arctic Seas to New Jersey, and from the Bering Sea to San Pedro, California (Abbott, 1974). Its global range includes the coasts of Japan, Iceland, Europe, northwestern Africa, and the Mediterranean countries (Madsen, 1949; Rowell, 1967). It is primarily a subtidal species, inhabiting rocky and gravelly bottoms to a depth of 280 meters (Wiborg, 1946; Madsen, 1949; Rowell, 1967).

The spawning season of *M. modiolus* is ill-defined and may vary greatly with depth and geographical location. It has been suggested that major spawnings occur at intervals of several years, with the possibility of partial spawnings one or more times each year (Wiborg, 1946; Rowell, 1967). Release of gametes has been observed from February through July in European waters (Nordgaard, 1901; Williamson, 1907; Wiborg, 1946).

Early efforts to establish the identity of *M. modiolus* larvae in the plankton were inconclusive, and as Jørgensen (1946, p. 289) states, "the resemblance to the *Mytilus edulis* veliger may be so great that a confusion of these two species may have taken place." Although Rees (1950) and Newell and Newell (1963) compared the larval forms of these two closely related mytilids, their identification of *M. modiolus* relied on the error-prone "indirect method" (see discussion in Loosanoff, Davis, and Chanley, 1966), and subtle differences in shape, color, and texture constituted the only distinguishing features between the two veligers.

Laboratory work with larval *M. modiolus* has been nearly nonexistent. Nordgaard (1901) carried larvae through the straight-hinge stage, but inadequate culture techniques prevented the observation of further development. Williamson (1907) witnessed a natural spawning of adult horse mussels in the laboratory but made no attempt to rear larvae. The present study provides a detailed description of laboratory-cultured *M. modiolus* larvae from the straight-hinge stage to metamorphosis, including photographic and statistical comparisons with *Mytilus edulis* L. to aid in differentiating the two species.

MATERIALS AND METHODS

Sexually mature adult horse mussels were collected subtidally along the coast of Chamberlain, Maine, and were placed in a running saltwater bath at approximately 14° C. Attempts to spawn the mussels by transferring them to a warm (20° C) water bath, by rapidly fluctuating the water temperature, and by adding stripped gametes to the water were generally unsuccessful. The few fertilized eggs thus obtained showed slow and abnormal development. Thermal stimulation followed by individual injections of 0.5 M KCl, as described by Bayne (1965), also proved ineffective in inducing release of gametes. On one occasion, however, spawning occurred spontaneously when the water flow through the holding tank was temporarily stopped.

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Eggs from this spontaneous spawning were transferred to a 55 l polyethylene tank, which was filled with sea water filtered to remove particles 1 μ or larger in diameter. Numerous eggs were also pipetted along with culture water onto a glass slide (no coverslip) and photographs taken for subsequent measurements of fertilized egg diameters. After 72 hrs larvae were transferred to a 16 l polyethylene container and adjusted to a low density (1–5/ml) to minimize growth rate reductions due to crowding. The temperature of the culture ranged between 16° and 21.5° C, and salinity varied little from 30‰. Filtered sea water was used to change the cultures every other day, at which times larvae were collected for preservation in 95% EtOH. Larvae were fed daily with concentrations of *Isochrysis galbana* Parke ranging between 30,000 and 80,000 cells per ml of culture. Techniques for handling eggs and larvae followed closely those described by Loosanoff and Davis (1963).

Several adult specimens of *M. edulis* were conditioned and spawned according to the procedure outlined by Bayne (1965), and cultures of these larvae were also carried through metamorphosis using the techniques of Loosanoff and Davis (1963). Larvae were sampled every other day and preserved in 95% EtOH.

The micrographs and length-height measurements for this study were obtained from whole larvae, taking care to achieve consistent and representative larval orientations. Obtaining such consistent orientations, however, is rendered difficult by the convex shape of intact specimens. Nevertheless, dissociating the two valves is a tedious process and most routine plankton identifications will utilize only the intact bivalve. Loosanoff, Davis, and Chanley (1966) have discussed problems associated with the identification of bivalve larvae, including the relative merits of using whole larvae or single valves in photomicrograph sequences and the worth of various dimensional measurements.

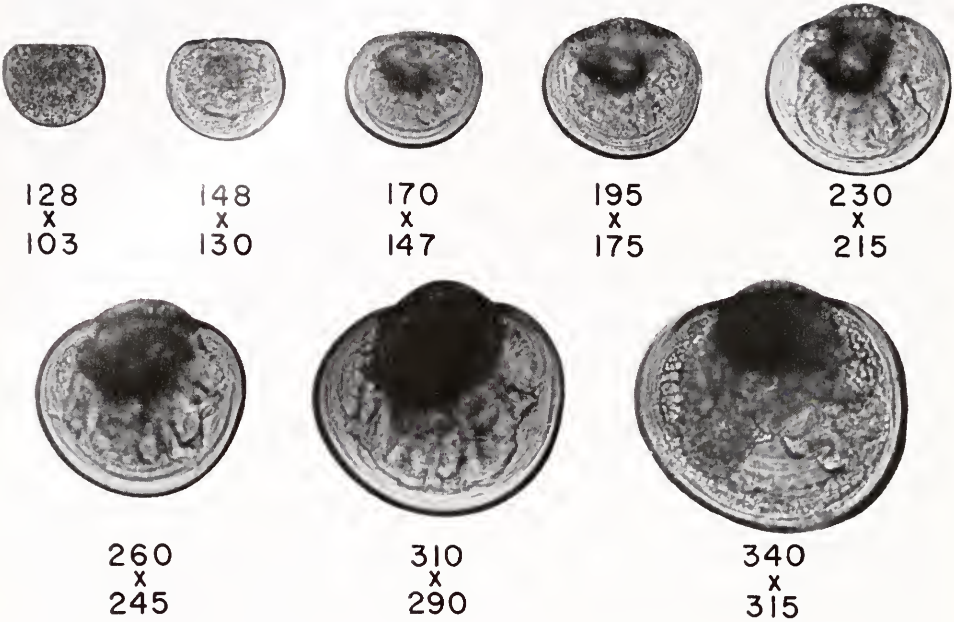
Preserved larvae of both species were measured to the nearest 5 μ with a calibrated ocular micrometer, and values for hinge-line length, total length, height, and depth were obtained. Three-dimensional growth diagrams were constructed using the method described by Chanley and Van Engel (1969). A Cambridge S-4 scanning electron microscope was used to examine the surface shell morphology of individuals from the straight-hinge stage through metamorphosis. Descriptive terms are defined in Chanley and Andrews (1971).

RESULTS

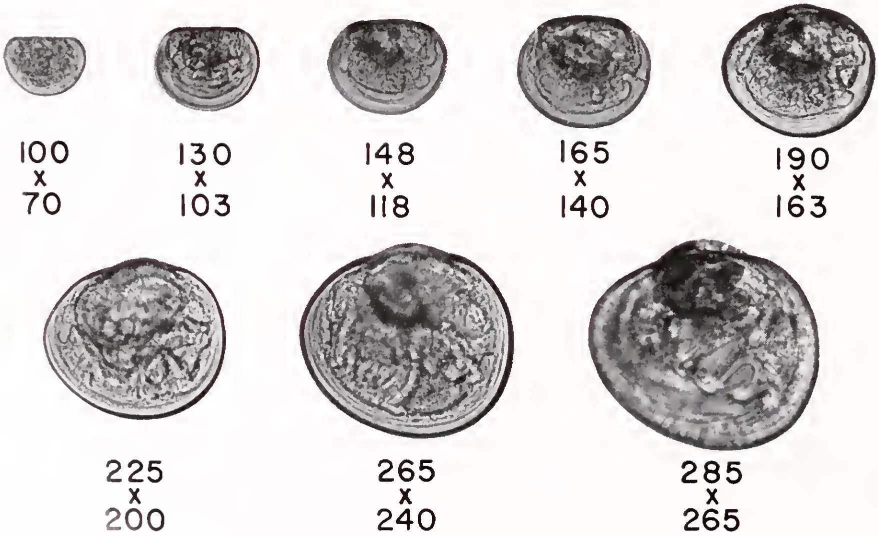
Larval development of Modiolus modiolus

Fertilized eggs measured from 78 to 90 μ in diameter with a mean of 85 μ. Development through the ciliated gastrula and trochophore stages was similar to that described by Field (1922) for *M. edulis*. The elongate, somewhat conical trochophore displayed a prominent apical flagellum on its larger end.

Modiolus modiolus


128 x 103
148 x 130
170 x 147
195 x 175
230 x 215
260 x 245
310 x 290
340 x 315

Mytilus edulis


100 x 70
130 x 103
148 x 118
165 x 140
190 x 163
225 x 200
265 x 240
285 x 265

The smallest shelled veliger observed had length, height, and straight-hinge dimensions of 105, 90, and 95 μ, respectively. The distribution of hinge-line lengths remained constant over time, with a mean of 100 μ and extremes of 90 and 110 μ. As total lengths reached 150 to 165 μ the hinge-line was obscured by the appearance of a low, rounded umbo, which subsequently developed into a knobby protrusion (Figs. 1, 2). As with most mytilids (Chanley, 1970), the anterior end of umbo stage individuals was more pointed than the posterior end.

Measurements of 745 larvae from early straight-hinge through the pediveliger stage were used to run a linear regression of height on length, giving the relationship: height = 1.04 length − 29.26 ($r^2 = 0.98$). The same calculation for 89 length *versus* depth observations yielded the equation: depth = 0.67 length − 16.90 ($r^2 = 0.97$). In Figure 3 these length-height and length-depth data are combined to give an approximate three-dimensional representation of minimum and maximum sizes over the entire larval period.

The apical flagellum was still visible on the velum of the shelled larvae up to total lengths of 260 μ (Fig. 5B); whether or not it was present beyond that point was not ascertained. As larvae attained lengths of 230 to 245 μ, the nascent larval foot was evident posteriorly within the shell. Pigmented eyespots began appearing as larvae exceeded 270 μ in size, and the full-grown foot of the pediveliger stage became functional as larvae surpassed 295 μ in length. Eyespots measured 6 to 10 μ in diameter.

Scanning electron micrographs of the exterior hinge region (Fig. 2) reveal no distinctive features such as taxodont teeth or asymmetry of the left and right valves. Lack of growth in hinge length, however, indicated previously by near-constant straight-hinge measurements, is further evidenced by the clear delimitation of the hinge structure at either end of Prodissoconch I. Similar micrographs of the surface of the left valve (Fig. 2) demonstrate the sharp boundary between Prodissoconchs I and II as well as the concentric ridges of Prodissoconch II.

Although the culture was not maintained at a strictly constant temperature, times for the development of certain larval features in *M. modiolus* at culture temperatures of 16–21° C can serve as a general basis for comparison with other bivalve larvae. Day of fertilization equals day zero. Approximate time to the first straight-hinge larvae is 40 hrs; rounded umbo, 8 days; knobby umbo, 13 days; developing foot visible inside shell, 13 days; eyespot, 17 days; active foot, 18 days; first settled larvae, 19 days.

On any given day the culture contained a wide range of larval sizes, and the extent of the size range increased with time. At 2 days shell lengths were evenly distributed between 105 and 140 μ, a spread of 35 μ. At 11 days lengths varied over a range of 60 μ, and by 17 days larvae differed in length by as much as 100 μ.

Larval development of Mytilus edulis

The diameter of fertilized eggs averaged 67 μ. Observations on cleavage and embryological development up to the shelled veliger stage agreed closely with the

FIGURE 1. Photomicrographs of the larvae of *Modiolus modiolus* and *Mytilus edulis* from the early straight-hinge stage to metamorphosis. Length and height dimensions are given in microns below the individual specimens. Larvae are oriented with the anterior end to the left.

comprehensive description given by Field (1922). The trochophore possessed a distinct apical flagellum which persisted throughout subsequent larval development.

Measurements of newly-shelled straight-hinge larvae gave minimum length, height, and hinge-line dimensions of approximately 95, 70, and 70 μ, respectively.

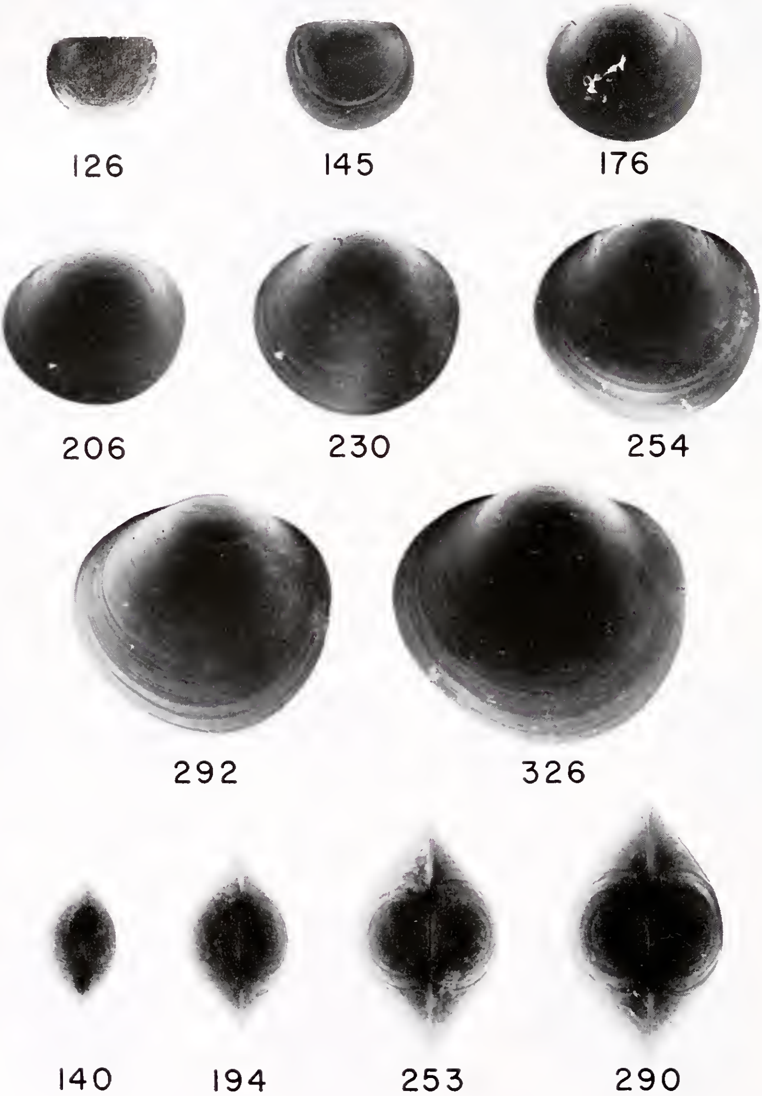


FIGURE 2. Scanning electron micrographs of the larvae of *Modiolus modiolus* from early straight-hinge stage to metamorphosis. Photographs in the top three rows depict the shell surface morphology of the left valve, while those in the bottom row represent a dorsal view of the hinge region. Length dimensions are given in microns below each specimen.

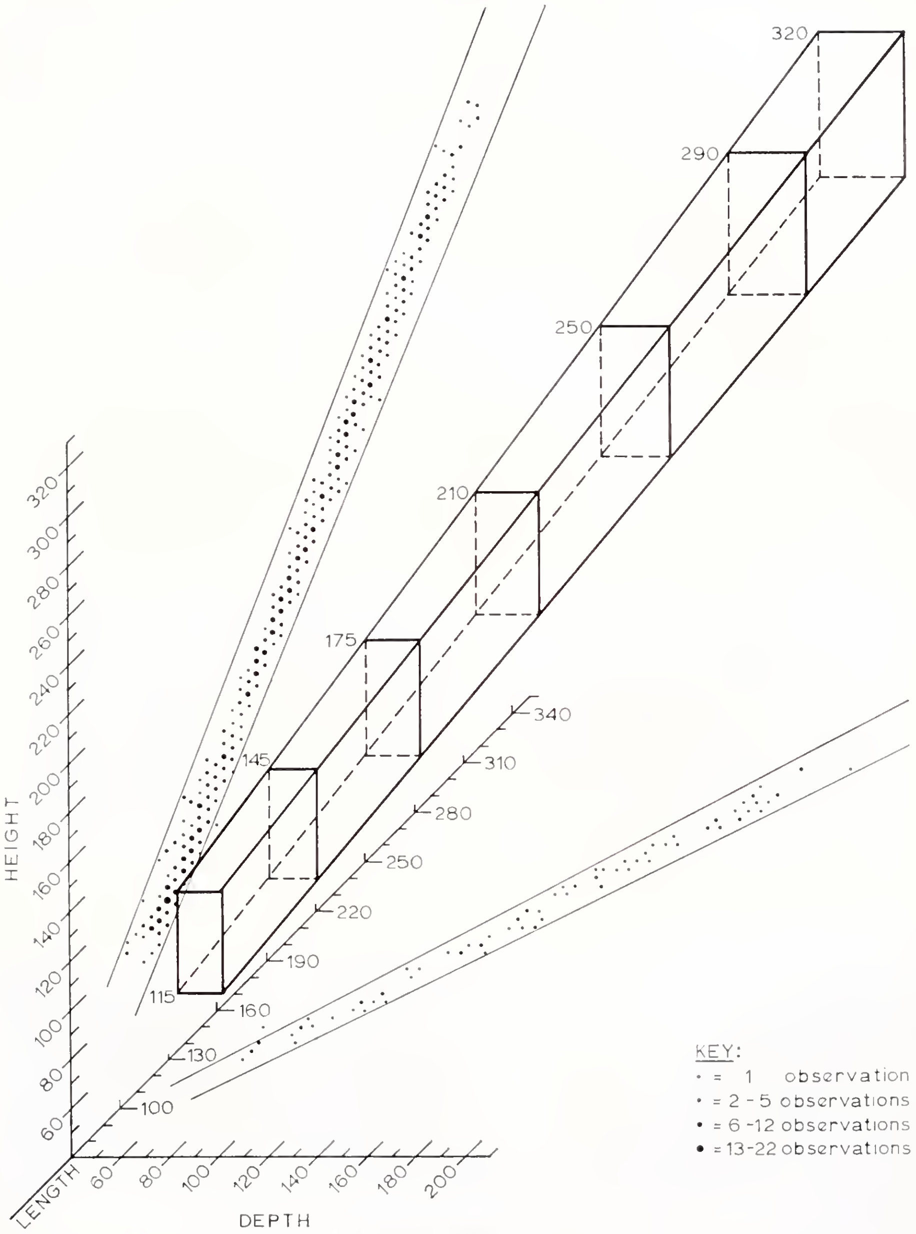


FIGURE 3. Larval dimensions of *Modiolus modiolus*. Height and depth coordinates run parallel to the length axis. Dots represent observed length-height or length-depth measurements. The polyhedron was constructed according to the procedure outlined by Chanley and Van Engel (1969) and encompasses all possible length-depth-height combinations of *M. modiolus* larvae.

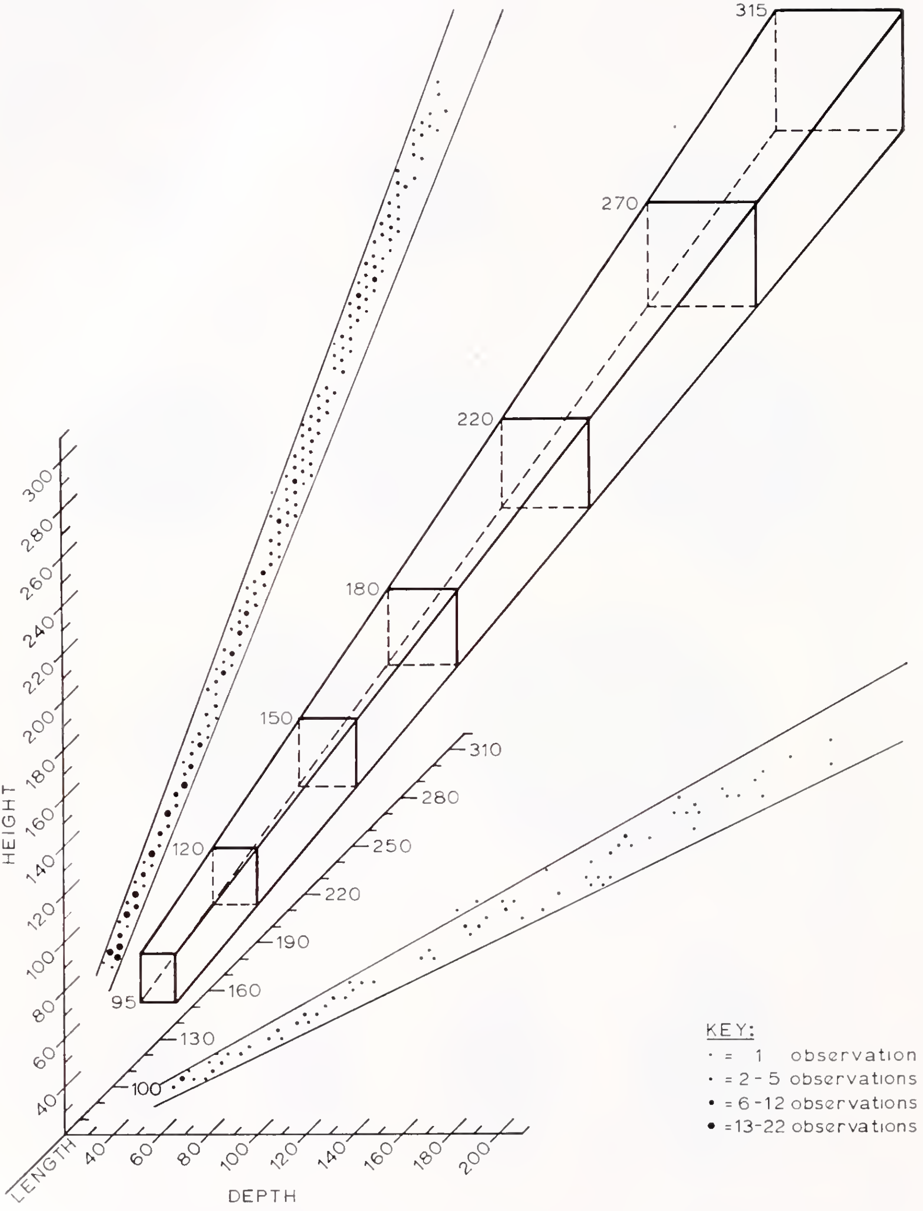


FIGURE 4. Larval dimensions of *Mytilus edulis*. Height and depth coordinates run parallel to the length axis. Dots represent observed length-height or length-depth measurements. The polyhedron was constructed according to the procedure outlined by Chanley and Van Engel (1969) and encompasses all possible length-depth-height combinations of *M. edulis* larvae.

Hinge-line lengths ranged from 70 to 80 μ, with the mean remaining constant over time. When larvae reached 140 to 150 μ in total length, the appearance of a rounded umbo marked the transition from the straight-hinge to the umbo stage of development. As larvae grew past 210 to 230 μ in length, the umbo gradually extended from the hinge and shoulders as a low knob (Fig. 1).

A linear regression of height on length, run on data from 550 *M. edulis* veligers in all stages of development, yielded the equation: height = 1.08 length − 37.22 ($r^2 = 0.99$). Eighty-eight length *versus* depth measurements were used in a similar fashion to calculate the equation: depth = 0.61 length − 15.82 ($r^2 = 0.96$). The length-height and length-depth data were then combined to create a three-dimensional approximation of maximum and minimum larval sizes for the entire shelled veliger stage (Fig. 4).

Eyespots were detected in larvae as small as 205 μ long, although the majority developed them when shell lengths reached 220 to 230 μ. Larvae as large at 245 μ were occasionally observed to lack an eyespot. The eyespot diameter varied from 5 to 7 μ. A well-defined foot could be seen in larvae 195 to 210 μ in length, and active extension of this organ began in larvae between the lengths of 215 and 240 μ.

As with *M. modiolus*, scanning electron micrographs of the exterior hinge

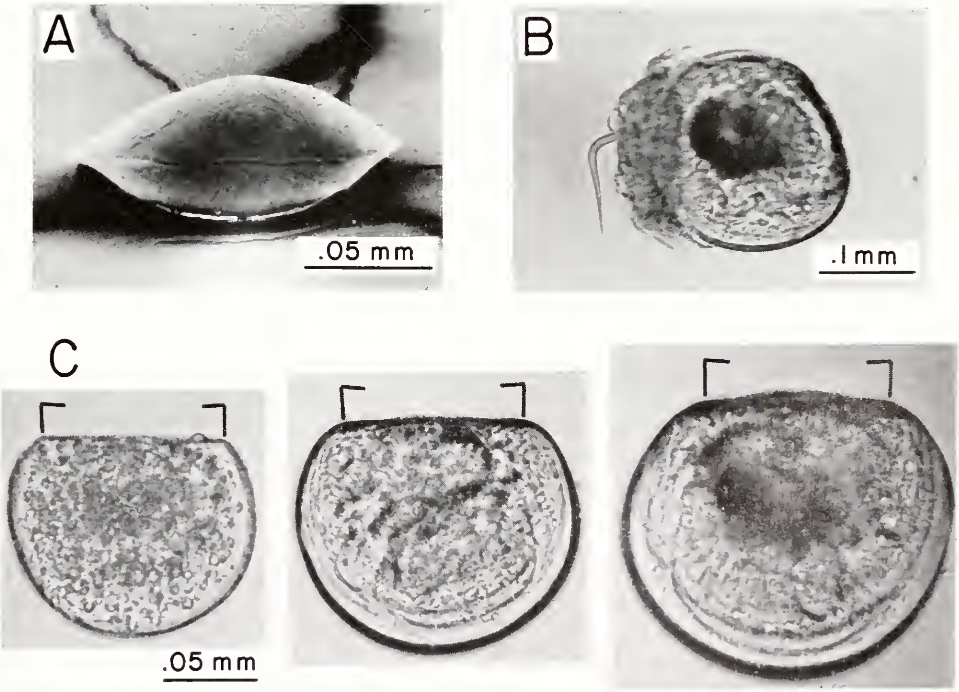


FIGURE 5. (A) Scanning electron micrograph of the hinge region of a straight-hinge veliger larva of *Mytilus edulis*; (B) early umbo stage larva (*Modiolus modiolus*) showing prominent apical flagellum; (C) various stages of development of straight-hinge larvae of *Modiolus modiolus*. Brackets delimit the "hinge-line" as measured in this study.

region revealed no asymmetry of the left and right valves, nor did they show any external manifestation of hinge teeth (Fig. 5A).

DISCUSSION

Obtaining viable sperm and eggs appears to be the greatest barrier to more extensive work with *M. modiolus* larvae in the laboratory. The natural spawning which was observed upon cessation of the water current in the holding tank is identical to an experience reported by Williamson (1907) in his work with *M. edulis.* Subsequent attempts to reproduce this phenomenon and to thus establish a reliable means of spawning the horse mussel proved ineffective. Since thermal, chemical, and sex product stimulation of sexually mature individuals also failed to induce spawning in this species, exploration of other methods would be highly desirable. Electrical stimulation (Iwata, 1950; Sugiura, 1962) and pricking of the posterior adductor muscle (Loosanoff and Davis, 1963) have successfully induced release of gametes in *M. edulis,* while artificial fertilization of stripped eggs and sperm has resulted in healthy larvae in some species (Loosanoff and Davis, 1963). Should these methods prove ineffective in themselves, long-term thermal conditioning may render the adults more susceptible to such stimuli.

The fertilized egg diameter range (78–90 μ) for *M. modiolus* observed in this study, is compatible with Williamson's (1907) reported value of 0.1 mm, as his value was only given to the nearest tenth of a millimeter. Wiborg (1946), however, gave a range of 0.09 to 0.14 mm, with an average of 0.10 to 0.11 mm. These measurements differ considerably from the observations made in this study. As Wiborg (1946) failed to state the conditions under which the egg diameters were measured, it is only possible to speculate on factors responsible for the observed differences. The presence or absence of a coverslip or osmotic swelling or shrinkage due to different salinities during measurement might account for the discrepancies. It is also possible that the different reported diameters may be a morphological manifestation of subspecific variation between mussels from geographically isolated populations. A much more detailed study would be required to ascertain the existence of such subspecific differences.

The wide range of sizes for larvae of the same age and living in the same environment is a phenomenon common to many bivalve molluscs, as noted by Loosanoff and Davis (1963), and is most likely due to normal genetic variation. The point at which *M. modiolus* larvae reached a certain stage of development, however, seemed to be more dependent on size than on age. For example, the first larvae to possess an eyespot or an active foot were also the largest larvae in the culture on that day, the proportion of individuals possessing such characteristic features generally equaling the proportion of these larger individuals in the total population. Even when a sizable spatfall was visible on the container bottom, smaller individuals in the culture had not yet reached the pediveliger stage. Loosanoff and Davis (1963) and Loosanoff, Davis, and Chanley (1966) discussed evidence which shows that size at metamorphosis is independent of the geographical origin of the parents for several species of bivalve larvae, and independent of culture temperature for at least one species (*Mercenaria mercenaria* (L.)). Similarly, Bayne (1965), in his work with *M. edulis*, noted that individual larvae seldom reached the pediveliger stage before 250 μ. Although individuals show small variations in the size at which a characteristic feature appears, results such as these demonstrate the validity of correlating larval dimensions with stages of larval development for identification purposes.

Many authors have presented descriptions of the development and settling behavior of *M. edulis* (Stafford, 1912; Field, 1922; Werner, 1939; Sullivan, 1948; Bayne, 1964, 1965; Chanley and Andrews, 1971). Only data pertinent to a quantitative comparison with *M. modiolus* larvae were gathered for the present study. Williamson (1907) and Field (1922) gave values for the diameter of *M. edulis* eggs (0.06 to 0.07 mm and 0.07 mm, respectively) which agree well with the average value of 67 μ in this study. The minimum dimensions for straight-hinge veligers offered by Sullivan (1948) (length × width values of 155 × 120 μ) are much larger than those observed in this study but the smaller veligers could have been absent from her plankton tow samples. Data from the laboratory cultures of Chanley and Andrews (1971) are in close agreement with the data of this study, yielding minimum length, height, and depth measurements about 5 μ smaller and nearly identical observations on the lengths at which the umbo, eyespot, and pediveliger foot develop.

The results in this study suggest that *M. edulis* and *M. modiolus* straight-hinge veligers are readily distinguished by the difference in their hinge-line lengths. Both species maintained a constant distribution of hinge-line lengths over time, with the *M. edulis* hinge averaging 74 μ long and that of *M. modiolus* averaging 100 μ long. The largest hinge measurement for *M. edulis* was 80 μ and the smallest for *M. modiolus* was 90 μ, making this a definite means of differentiating between younger larvae of the two species. This conclusion, however, should be viewed with a certain degree of caution. Hinge-line lengths of *M. edulis* larvae recorded in this study are restricted to a somewhat narrower range than the 65–85 μ range reported by Chanley and Andrews (1971). In addition, Chanley (1970) and Chanley and Andrews (1971) reported a slight increase in the length of the hinge-line of most mytilids (including *M. edulis*) with larval growth. Such an increase was not observed for either of the species examined in the present study. The differences between their results and those of the present study can be accounted for by their inclusion of the straight dorsal extremities of the larval shoulders in their "straight-hinge" measurements of larger larvae (Chanley, personal communication). Actually the hinge-line is a Prodissoconch I character and, as with other bivalves, does not change with larval growth. In order to adequately distinguish this hinge-line from the dorsal extremity of each shoulder of relatively large *M. edulis* and *M. modiolus* larvae, it is necessary to focus up and down with the microscope until the hinge itself is in the plane of focus. Under such viewing conditions, the outside boundaries of the hinge-line generally coincide with the points at which the relatively flat dorsal region of the shell begins to gradually slope ventrally. It is recommended for future consistency that the "hinge-line" be considered as the dorsal region of the shell between these points. Representative photographs of several straight-hinge stages (*M. modiolus* in this case) are marked in Figure 5C in an attempt to delimit the "hinge-line" as defined in this manner. Determining the points at which the shell begins to slope ventrally (even though the change in slope may be very slight) is felt to be much less subjective than attempting to pinpoint the outside boundaries of the hinge-line at specific places along the constantly sloping shoulders of the dorsal region. Werner (1939), in his work with North Sea plankton samples, reported an average hinge-line length for *M. edulis* of 93 μ. While such a measurement may reflect a morphological (perhaps subspecific) variation between North Sea *M. edulis* populations and those of the western North Atlantic, it is probably more reasonable to conclude that some of Werner's larval identifications, being based upon the "indirect method," were in error.

Distinguishing small *M. edulis* and *M. modiolus* straight-hinge veligers from one another is also possible through a comparison of total shell lengths. The smallest *M. modiolus* larvae observed measured 105 μ long by 90 μ wide, a clear 10 μ longer than the smallest *M. edulis* veliger. A simple length measurement in the 95 to 105 μ range can eliminate confusion between these two mytilids.

A comparison of the *M. edulis* and *M. modiolus* length-height scatter diagrams (Figs. 3, 4) shows them to be very similar. The length-depth measurements (Figs. 3, 4), however, demonstrate the fact that *M. modiolus* larvae (regression equation: depth = 0.67 length − 16.90) are generally of greater depth than *M. edulis* larvae (regression equation: depth = 0.61 length − 15.82). This fact should be used with caution in larval identification, since the larger *M. edulis* depth measurements at a given length often extend into the range of the corresponding *M. modiolus* depth sizes.

Careful examination of shell shape is also helpful in discriminating the larval forms of these two closely related Mytilidae. As noted by Newell and Newell (1963), the umbo of *M. modiolus* is generally more prominent than that of *M. edulis* for larvae of comparable sizes. In addition, at lengths greater than 200 μ, larvae of *M. modiolus* present a much more rounded appearance than those of *M. edulis*, with the anterior end of the latter being considerably more pointed than that of the former. This observation is diametrically opposite of that reported by Rees (1950) and casts doubt upon the accuracy of his indirect larval identifications of the two mytilids.

In the present study the larval stages of *M. modiolus* were found to be more opaque than those of *M. edulis*. As the shell of *M. modiolus* is generally of greater depth than that of *M. edulis*, it is suggested that the more opaque appearance of *M. modiolus* may be a reflection of relatively greater thickness of soft body parts.

As evidenced by examination of the scanning electron micrographs presented in this study, examination of the external hinge structure is of little or no value in distinguishing the two species. While such a discrimination may well be possible through careful examination of the internal hinge structure, the removal of individual valves is tedious and usually impractical for routine identifications in plankton samples (Chanley and Andrews, 1971).

In plankton samples containing live or well-preserved larvae, the presence of an eyespot in individuals with lengths less than 270 μ may be helpful in distinguishing *M. edulis* larvae from those of *M. modiolus*. In *M. edulis*, eyespots were observed in larvae as small as 205 μ, while such structures were not seen in *M. modiolus* until the larvae had attained a size of 270 μ.

The presence of a well-developed larval foot should also aid in discriminating the two species. In *M. modiolus*, the full grown foot of the pediveliger stage does not appear to become active until the larvae attain a length of at least 295 μ. In contrast, the larval foot of *M. edulis* was observed to be functional in larvae with lengths as small as 215 μ.

In the present study larvae of *M. modiolus* were found to attain a much greater length than those of *M. edulis* prior to settlement and metamorphosis. Maximum shell length of the larvae, however, is probably of little aid in distinguishing the two mussels in the plankton. Bayne (1965), while studying the delay of metamorphosis of *M. edulis* larvae, found a significant correlation between mean maximum size and temperature, encountering larval *M. edulis* with lengths up to 415 μ. It is reasonable to assume that *M. modiolus* may also be capable of achieving such lengths (perhaps even greater) under various environmental conditions.

While the present study has stressed a comparison between *M. modiolus* and *M. edulis*, the data presented should be of assistance in distinguishing these species from other mytilids. A comprehensive summary of larval characteristics of the Mytilidae is presented by Chanley (1970).

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SUMMARY

1. Spontaneous spawning of *Modiolus modiolus* occurred under laboratory conditions at a temperature of approximately 14° C, and larvae were successfully cultured through settlement and metamorphosis.

2. Fertilized egg diameters varied from 78 to 90 μ with a mean of 85 μ.

3. The smallest shelled veliger observed had length, height, and straight-hinge dimensions of 105, 90, and 95 μ, respectively. The distribution of hinge-line lengths remained constant over time, with a mean at 100 μ and extremes of 90 and 110 μ.

4. As total lengths reached 150 to 165 μ the hinge-line was obscured by the appearance of a low, rounded umbo which subsequently developed into a knobby protrusion.

5. Pigmented eyespots began appearang as larvae exceeded 270 μ in size, and the full-grown foot of the pediveliger became functional as larvae surpassed 295 μ in length.

6. Discrimination of *Modiolus modiolus* and *Mytilus edulis* veligers is generally possible through a comparison of the following larval characteristics: hinge-line lengths, total shell length in the 95 to 105 μ range, shell shape of umbo stage larvae, presence of an eyespot in specimens with lengths less than 270 μ, and presence of a functional foot in larvae less than 295 μ.

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SOCIAL ORGANIZATION WITHIN CLONES OF THE SEA ANEMONE *ANTHOPLEURA ELEGANTISSIMA*

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The aggregating form of the anemone *Anthopleura elegantissima* often lives attached to large boulders and rocky outcroppings in closely packed groups. These are composed of genetically identical individuals, the products of asexual reproduction (Francis, 1973a). Contact between the tentacles of genetically different (non-clonemate) members of the species elicits aggression in one or both animals, while similar contact between clonemates is tolerated passively. Clonal aggregations remain separated from each other (Fig. 1) at least partly as a result of intraspecific aggression occurring at their adjacent borders (Francis, 1973b).

This aggressive behavior is rather elaborate, involving inflation of specialized effector organs, the blunt, white-tipped acrorhagi (Fig. 2), and stretching and bending movements of the oral disc and column that bring these turgid acrorhagi into contact with the non-clonemate adversary. During contact, scraps of the white acrorhagial ectoderm are applied to the body of the adversary where they adhere, slowly firing their batteries of large atrich nematocysts. Both the acrorhagi themselves and their large atrich nematocysts are deployed only during aggression (Äbel, 1954; Bonnin, 1964; Francis, 1973b). For an animal with a relatively limited behavioral repertoire and quite limited specialization of tissue types, this extensive investment in intraspecific aggression is remarkable.

In large clonal aggregations relatively few of the anemones are in a position to engage in battles at any given time. One might therefore expect the costs of intraspecific aggression to be distributed unevenly in such groups, with those animals at or near an interclonal border bearing more of the defense costs. To investigate this, I collected anemones from large, compact aggregations and compared individual size, sexual and asexual reproductive state, and acrorhagus size and number for animals from different locations within each clonal aggregation.

MATERIALS AND METHODS

Collection, holding and anesthetization of specimens

The anemones were collected at low tide by gently prying the pedal discs free of the rock substratum. At Scripps Institute of Oceanography (SIO) and at the University of Washington's Friday Harbor Laboratories, the anemones were kept in bowls supplied with flowing sea water. They were anesthetized by replacing about half the sea water in each bowl with a 7% solution of magnesium sulphate, which was merely poured into the bowls. The anemones remained fairly well expanded during this process and most of them remained expanded without aeration or exchange of water during the two to twelve hour anesthetization period when the bowls were kept at ambient seawater temperature. After anesthetization the anemones were removed and examind individually.

Counting and measuring acrorhagi

The fine fire polished tip of a bent pasteur pipette was used to fold back the limp tentacles and capitulum of the anesthetized anemones, exposing the acrorhagi which lie in the channel-like fosse at the top of the column. The total number of acrorhagi was recorded for each individual collected.

Acrorhagus size was determined for some of these anemones. If an anemone had fewer than 25 acrorhagi, all of these were removed and measured. If it had more than 25 acrorhagi, sometimes all, but usually every fourth one was removed and measured. The acrorhagi were first excised using iridectomy scissors and then placed on a glass slide with enough anesthetizing medium to barely float the coverslip. Because of their shape (Fig. 2) the acrorhagi always flattened laterally beneath the covership, with folds parallel to their long axes. The widths at the widest part of the white tip and the lengths from the acrorhagus tip to the base of the atrich-bearing ectoderm were measured at 350 ×.

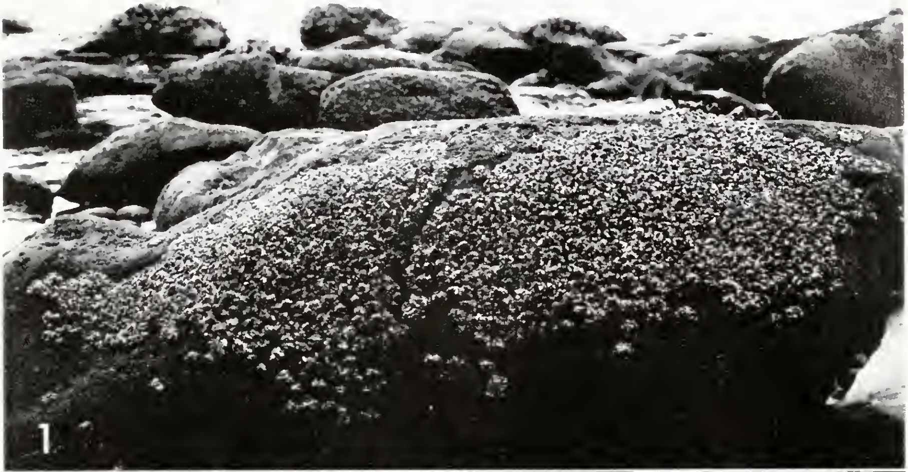
There are several reasons for measuring only the nematocyst-laden tips of the acrorhagi: first, this is the area actually involved in inflicting damage during aggression; secondly, there is no other discrete division between the acrorhagus and the fosse; and thirdly, the basal portion of the acrorhagi is very thin and extensible, stretching greatly with an increase in internal fluid pressure (Fig. 2) and wrinkling and folding more after excision than the thicker ectoderm at the tip. The flattened tips of the acrorhagi are roughly elliptical, and acrorhagus area is accordingly approximated as twice the area of an ellipse with the major and minor axes measured as described above.

The photographs (Fig. 2) were taken in a tidepool at Eagle Point, Washington. To initiate the aggressive response, the tentacle tips of the anemones were repeatedly touched with a tentacle excised from a non-clonemate. The anemones were photographed at the same phase of the response (with the oral disc raised during the movement of application; Francis, 1973b) and are shown to the same scale (approximately 2.5 ×).

Reproductive state

Anemones were collected in the SIO preserve area and examined for the presence of gonads in mid-June (1975) when the gonads should be well developed (Ford, 1964). While the anemones were anesthetized, the presence or absence of gonads was determined by making a long incision in the surface of the pedal disc and examining the exposed mesenteries. Female gonads were brownish pink and male gonads were yellowish white. When uncertain, I excised pieces of mesentery and examined them microscopically for the presence of gametes. Most of the animals treated in this way recovered completely when returned to flowing sea water.

These anemones also reproduce asexually by longitudinal binary fission, and one often finds on their columns vertical scars that probably represent incomplete



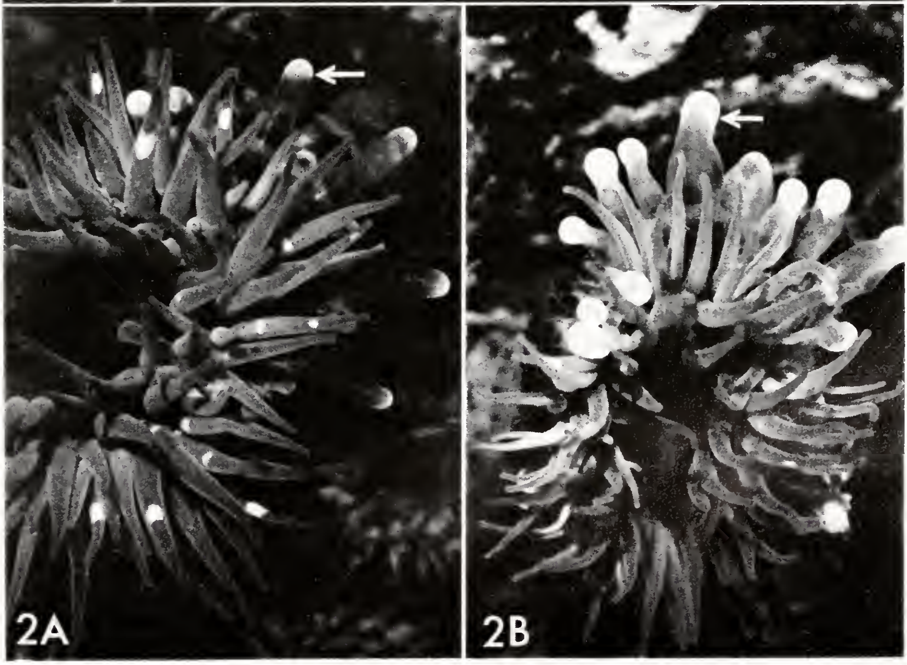


Figure 1. Boulders surrounded by sand (March, 1975) in the intertidal, Scripps preserve area, La Jolla, California. In the foreground clonal aggregations 201 and 202 are separated by a conspicuous anemone-free space. The other borders of the aggregations are free of intraspecific competition.

Figure 2. Two genetically identical anemones (approximately 2.5 ×) performing the aggressive response in a tidepool at Eagle Point, San Juan Island, Washington. The fully inflated acrorhagi (arrow) of the midclonal anemone (A) are much smaller and more sparse than those of a much smaller clonemate from an interclonal border (B).

healing of a relatively recent division. The animals collected to assay gonad development were also examined for the presence of fission scars.

Wet weight determination

Wet weight was used as an index of individual size that is independent of differences in body composition and readily determined without damage to the animals. To minimize random variation in the fluid content of the coelenteron, the anesthetized anemones were gently squeezed and blotted with damp absorbent paper. They were then weighed to the nearest 0.1 g on a top-loading balance.

Sampling

Comparing animals from different locations within the same clonal aggregation eliminates variation due to genetic differences and isolates positional effects. Samples from each clone included: all of the animals from a border adjacent to another clonal group (the interclonal edge); at least an equal number of individuals from a border roughly parallel with the interclonal edge and free of contact with nonclonemate conspecifics (the free edge); and at least as many anemones taken in rows roughly parallel to the interclonal border and a minimum of 10 cm from either the free border or the interclonal border (midclone). To avoid differences in the period of daily tidal exposure and/or seasonal sand burial between the sample groups within each clone, care was taken to collect all three samples from approximately the same tidal heights. To assure that the sampling was not biased, the clones were chosen and the samples collected during tidal exposure when the animals were contracted, making it impossible to see the acrorhagi. Unless specifically noted, all of the data collected are reported here.

Most of the samples were collected at La Jolla, California in the Scripps Institute of Oceanography (SIO) preserve area between the Scripps pier and Black's Beach. This area was particularly suitable because the clonal aggregations meet the criteria outlined below, being closely packed and isolated on the open faces of large boulders that are surrounded by sand (Fig. 1). For comparison, samples were also taken in Puget Sound at Eagle Point on San Juan Island, Washington, where the anemones are limited to crevices and tidepools, rather than colonizing boulder faces.

Several criteria were used in selecting the clones to be sampled. The clonal aggregations were as large as possible and quite tightly packed. Both of these circumstances minimize the likelihood of mixing between the groups sampled within each clone and thus accentuate positional differences. Further, the aggregations were relatively isolated from neighboring clones, except at one (SIO preserve area clones 201, 205, 209 and 223; and Eagle Point clone 223) or more (SIO preserve area clones 213, 216, 218, 221 and 222) well defined interclonal borders no more than 3 cm wide. This increases the likelihood that interclonal contact was occurring and had been limited to a narrowly defined area of the group. Finally, whenever possible the aggregations selected had an unobstructed, competition-free edge (*i.e.,* an edge of the clonal aggregation roughly opposite the interclonal border and free of neighboring conspecifics).

Table I

Relationship of individual size to position within the aggregation: mean wet weight (grams) ± their standard errors, and sample size.

Identifying number for each clone and (sampling date)	Position within the aggregation		
	Midclone mean ± s.e. (N)	Free edge mean ± s.e. (N)	Interclonal edge mean ± s.e. (N)
211 (IV/75)	0.74 ± 0.07 (10)	0.52 ± 0.04 (10)	none
221 (VI/75)	0.91 ± 0.09 (26)	none	0.48 ± 0.06 (16)
222 (VI/75)	1.12 ± 0.07 (20)	none	0.82 ± 0.11 (12)
223 (X/75)	0.54 ± 0.06 (27)	0.37 ± 0.05 (15)	0.38 ± 0.07 (27)
201 (III/75)	0.71 ± 0.08 (8)	0.50 ± 0.07 (8)	0.63 ± 0.12 (10)
205 (III/75)	1.11 ± 0.12 (30)	0.75 ± 0.08 (10)	0.74 ± 0.05 (10)
209 (III/75)	1.28 ± 0.20 (10)	1.04 ± 0.08 (9)	1.11 ± 0.14 (9)
216 (VI/75)	0.86 ± 0.16 (9)	0.69 ± 0.12 (7)	0.70 ± 0.16 (7)
grouped 201, 205, 209 & 216	1.01 ± 1.08 (37)	0.76 ± 0.05 (34)	0.79 ± 0.06 (36)

For comparison, samples were also taken from a clone apparently isolated from other conspecifics. SIO clone 211 rested in a shallow depression of a large, bare boulder; the nearest neighbors were 23 cm from the aggregation.

Results

Individual size and position within the aggregation

Wet weights are reported for anemones collected without size bias from at least two of the three standard locations (interclonal edge, free edge, and midclone) in eight separate clones. Some of the samples collected specifically to determine reproductive state were deliberately biased toward larger specimens, and those data are not included here.

Means and their standard errors for each group are shown in Table I. Since the samples taken from each clone were small, means and their standard errors were also calculated by grouping the data from the four La Jolla clones for which complete information is available (Table I). Sample sizes for the clones were similar except in one case; the midclone sample ($N = 30$) for clone 205 includes animals taken in three separate vertical rows. Only one (the most central) of these subsamples was included in calculating mean and standard error for the grouped data.

Number of acrorhagi per individual and position in the aggregation

Samples were taken at the standard locations in each clone and the anemones were returned to the laboratory where they were anesthetized and their acrorhagi

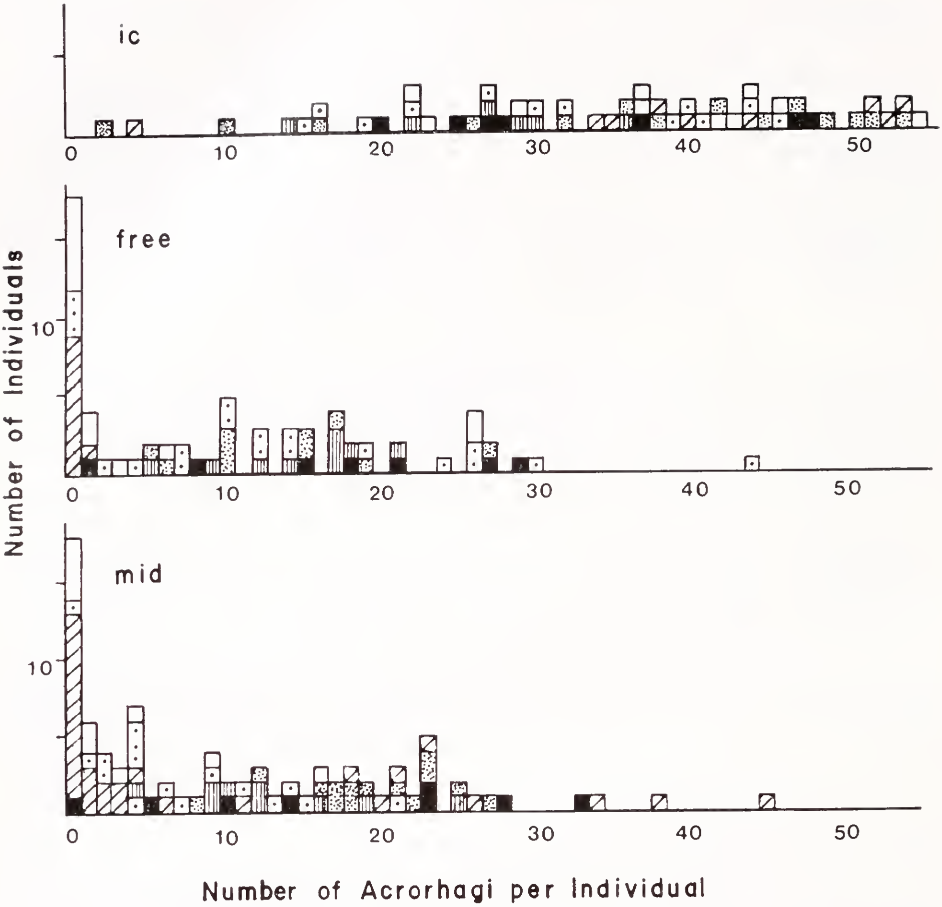


FIGURE 3. Number of acrorhagi per individual for anemones sampled from three different positions within each clone: an interclonal border (ic), a border free of intraspecific competition (free), and the middle of the clone (mid). The six clones sampled are clones 201 (dot), 205 (slash), 209 (open square), 213 (upright lines), 216 (black square), and 218 (stipple).

counted. The number of acrorhagi per individual is shown for each individual taken from the six La Jolla clones for which there is complete data (Fig. 3).

Means and their standard errors for these grouped data are shown in Table II. Since the samples from the clones were somewhat unequal, the means for each location in each of the six clones (Table II) were also calculated and from these, the mean of the means and their standard errors for each position: 1) midclone anemones, 11.1 ± 2.46, ($N = 6$); 2) free edge anemones, 10.6 ± 2.62, ($N = 6$); and 3) interclonal edge animals, 33.7 ± 1.43, ($N = 6$).

Also included in Table II are data from a small isolated clonal aggregation (without an interclonal border; clone 211) and two aggregations without competition-free borders (clones 221 and 222).

An appropriate correction for size variation could be made by dividing the number of acrorhagi per individual by the cube root of the individual wet weight. This correction greatly reduced the variance when applied to data collected from a large, apparently isolated aggregation from Eagle Point, Washington (unpublished data). Since the interclonal border anemones tended to be smaller than midclone anemones from the same clone (Table I), correction for size would emphasize the already apparent difference between the two groups. I have chosen instead to present the data in their simplest form.

Acrorhagus size and clonal position

Anemones from two of the clones sampled for acrorhagus number were also sampled to determine acrorhagus size. Differences in acrorhagus size are easily observed during the aggressive response (Fig. 2) and were readily apparent when the anesthetized anemones were examined to count their acrorhagi. However, the particular clones and individuals chosen for sampling and for the field photograph were selected haphazardly.

Figure 4 shows the area in mm^2 of all acrorhagi excised from anemones in the

TABLE II

Number of acrorhagi and individual position within the aggregation: means ± their standard errors and sample size.

Identifying number for each clone and (sampling date)	Position within the aggregation		
	Midclone mean ± s.e. (N)	Free edge mean ± s.e. (N)	Interclonal edge mean ± s.e. (N)
211 (III/75)	0.4 ± 0.16 (10)	4.2 ± 2.43 (10)	none
221 (VI/75)	6.6 ± 1.14 (19)	none	39.7 ± 3.93 (12)
222 (VI/75)	6.6 ± 0.99 (23)	none	27.8 ± 2.79 (16)
223 (X/75)	1.8 ± 0.65 (27)	2.6 ± 1.06 (15)	16.7 ± 2.56 (27)
201 (III/75)	6.6 ± 1.35 (14)	13.8 ± 3.25 (16)	31.0 ± 3.52 (11)
205 (III/75)	8.1 ± 2.38 (30)	0.1 ± 0.10 (10)	38.4 ± 4.67 (10)
209 (III/75)	3.4 ± 1.66 (10)	5.4 ± 2.82 (12)	35.1 ± 3.98 (9)
213 (VI/75)	13.7 ± 2.03 (10)	14.4 ± 1.67 (9)	28.6 ± 3.44 (7)
216 (VI/75)	15.6 ± 3.89 (9)	16.9 ± 3.89 (7)	33.1 ± 4.17 (7)
218 (VI/75)	19.2 ± 1.44 (14)	13.4 ± 1.60 (13)	35.7 ± 4.42 (14)
grouped 201, 205, 209, 213, 216 & 218	10.6 ± 1.13 (87)	10.6 ± 1.25 (67)	34.4 ± 1.71 (58)

various positions in both clones (open squares) and the mean acrorhagus tip area for each individual animal (large dots).

The mean acrorhagus size (acrorhagus tip area in mm^2) for each individual has been used to calculate means and their standard errors for each group: 1) clone 216—interclonal edge animals, 12.9 ± 2.34, (N = 7); 2) clone 216—free edge animals, 2.2 ± 0.60, (N = 6); 3) clone 222—interclonal edge animals, 11.6 ± 1.34, (N = 8); and 4) clone 222—midclone animals, 3.3 ± 0.55, (N = 8). Again these data are presented in their simplest form in preference to including a correction for the size variation among individuals.

Gonad development and individual position in the aggregation

Gonad development in this species is seasonal and size-related (Ford, 1964; Francis, 1973b); the animals were collected during the pre-spawning period and the data are reported as gonads present or absent for animals of specific sizes (wet weights). My own previous work as well as the present data clearly indicate that the lower size limit for sexual reproduction varies among clones. Larger samples

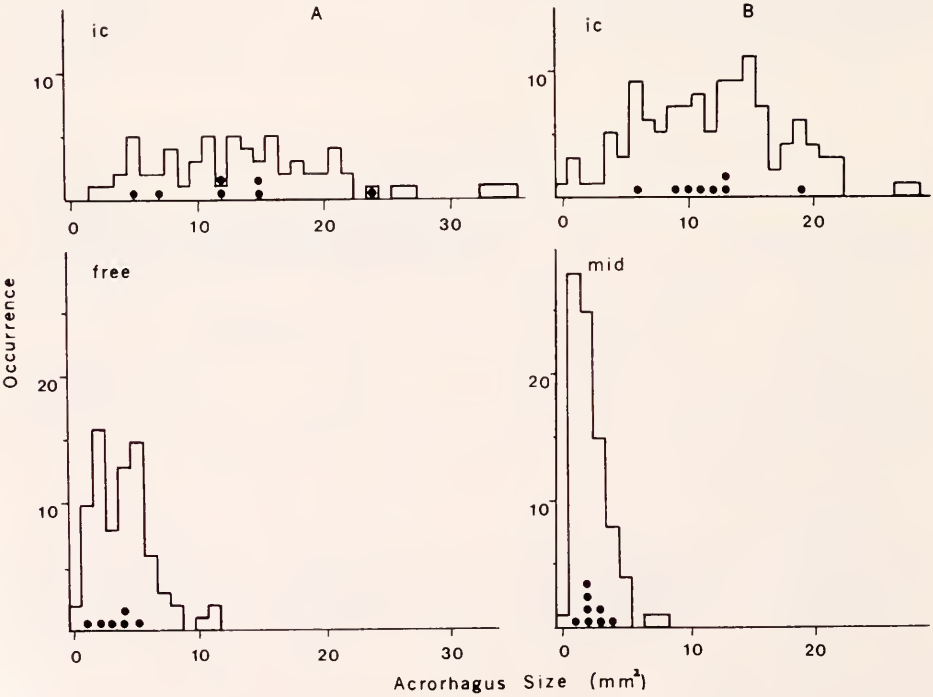


FIGURE 4. Size (mm^2) of each acrorhagus measured (bar graph) for anemones from different positions within clones 216 (A) and 222 (B); and average acrorhagus size per individual (dots) calculated from the same data. Anemones were collected from interclonal borders (ic), borders free of intraspecific competition (free), and the middle of the clones (mid).

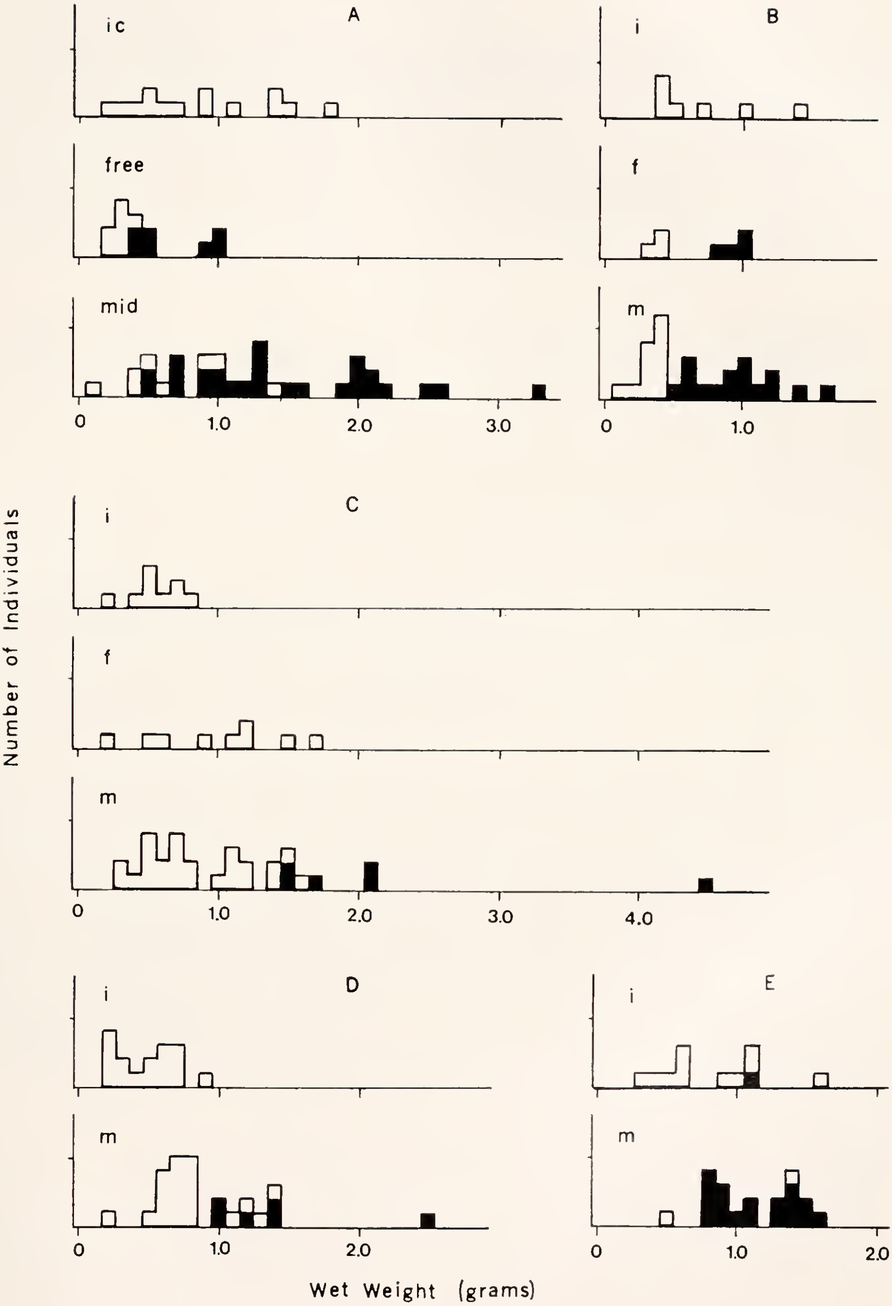


FIGURE 5. Presence of gonads (black square) or absence of gonads (open square) as a function of size (wet weight in grams) for anemones sampled from different positions (ic represents interclonal border; f, border free of intraspecific competition; and mid, midclone) within clone 218 (A), 216 (B), 213 (C), 221 (D), and 222(E).

Table III

Incidence of fission scarring and individual position within the aggregation: number of individuals scarred/number examined, and percent scarred.

Identifying number for each clone and (sampling date)	Position within the aggregation		
	Midclone	Free edge	Interclonal edge
221 (VI/75)	3/26 = 12%	none	0/12 = 0%
222 (VI/75)	1/20 = 5%	none	0/16 = 0%
213 (VI/75)	10/23 = 43%	5/9 = 56%	2/9 = 22%
216 (VI/75)	5/28 = 25%	1/7 = 14%	1/7 = 14%
218 (VI/75)	11/28 = 25%	5/11 = 45%	2/13 = 15%
grouped 213, 216 & 218	26/79 = 33%	11/27 = 41%	5/29 = 17%

were therefore taken from the middle of each aggregation to establish the limit for the clone, and the data for each clone is presented separately (Fig. 5). Samples from midclone and from the free edges of clones 213 and 218 were biased toward larger animals to increase the likelihood of collecting animals over minimum reproductive size. Of the five clones sampled only clone 222 was male; the rest were female.

Of the 42 anemones collected from interclonal borders, only one had gonads, and these were few and small. Eleven of the 46 free edge animals had developed gonads, and 73 of the 140 midclone anemones sampled had gonads. Since many of the edge animals were below minimum reproductive size, it is reasonable to compare the number of animals in each location that are above the minimum reproductive size for their clone. Of the 22 anemones from an interclonal edge that were larger than the smallest sexually developed individual collected from the same clone, only one had gonads. Eleven of the 14 anemones over minimum reproductive size from the free edge were ripe, and 73 of the 83 specimens over minimum reproductive size from midclone were ripe. A chi-square test using a two by three contingency table shows significance at above the 99.5% confidence level for either analysis.

Asexual reproduction and individual position in the aggregation

Table III shows the occurrence of fission scars on the columns of animals collected during June, 1975 and examined for scars. Complete information was available for three of the clones. When these data are grouped by position, a chi-square analysis using a two by three contingency table indicates that the apparent differences among the positional groups might be expected to occur by chance one time in twenty.

Inequalities in binary fission

"Daughter" pairs resulting from asexual divisions that occurred in the laboratory were weighed as soon as possible after fission to determine the equality of these divisions. The wet weight in grams for the larger of the two halves and the percent of the original individual that this represents are arranged here from the most to the least equally divided: 1) 0.86 g (51%); 2) 0.37 g (51%); 3) 0.31 g (53%) 4) 1.37 g (53%); 5) 1.06 g (55%); 6) 0.31 g (56%); 7) 2.64 g (58%); 8) 0.36 g (63%); 9) 7.22 g (63%); 10) 0.96 g (68%); and 11) 0.45 g (75%).

DISCUSSION

From these results it is apparent that the warrior anemones living at an interclonal border have more and larger acrorhagi than do their clonemates either at an edge free of intraspecific competition or in the midst of an aggregation. The striking lack of gonads among the warriors during the prespawning period might indicate that they spawn earlier than their clonemates or that they develop gonads and spawn later; however it seems more likely that border warriors usually fail to develop gonads at all. The rate of asexual reproduction (as indicated by the incidence of fission scars) may be lower among the fighting anemones than among the nonfighters, both those in the midst of aggregations and those at edges free of intraspecific competition. There is no apparent difference in the size of interclonal edge warriors as compared with free edge animals; however midclonal anemones are larger on an average than their clonemates at either edge of the group. All of these differences except the size difference are clearly related to the intraspecific aggressive behavior. Some of the implications of this situation bear further discussion.

For animals that reproduce asexually, the term *individual* is ambiguous; for a recent discussion, see Mackie, 1973. *Anthopleura elegantissima* reproduces asexually by binary fission, so one anemone is only temporarily distinguishable from its clonemates. It was formerly part of other now-fragmented anemones and will presumably continue to fragment indefinitely. With this explicitly understood, I shall continue to use the term *individual* to mean one temporarily distinguishable physical entity, the term *clone* to denote the genetic entity, and the terms *aggregation* and *clonal group* to identify a contiguous group of genetically identical anemones.

Without entirely sacrificing the genetic plasticity available through sexual reproduction, the clonal aggregating form of *Anthopleura elegantissima* has invested in an evolutionarily conservative life style involving longevity, indeterminate growth and asexual reproduction. For many organisms the sexual adult is a temporary vehicle for an evolutionarily plastic and potentially immortal genome. Longevity, and perhaps even immortality for the entire organism, is a reasonable strategy in a stable environment that is either unvarying or (like the intertidal) predictably varying. This requires that the organism be capable of extensive repair and regeneration.

Asexual reproduction in its simplest form, fragmentation, is also available only to organisms with a high capacity for repair and regeneration. With asexual reproduction, a genetic entity can grow indefinitely without the mechanical problems inherent in unlimited increase in individual size. Furthermore, a genotype dispersed as a number of separate individuals (*i.e.,* a clone) is less liable to be exterminated, a serious problem for the competitive immortal.

Unless external factors such as predation or environmental instability effectively limit population size, intense competition is inevitable among immortals that reproduce sexually and are capable of indeterminate growth.

There are two rather different ways to consider the connection between genetic similarity and intraspecific interactions. It is reasonable to expect that the more closely related two individuals are, the more similar their requirements will be, and the more extensive their competition for resources in a resource-limited situation. On the other hand, if it is of advantage to cooperate with conspecifics, cooperation with a close relative will be especially advantageous evolutionarily because any benefits gained in this way increase the fitness not only of the genes that the individual itself carries but also of the common genes carried by the near relative.

Whether an animal responds to conspecifics in an interfering or a cooperative manner will depend on whether the advantages gained by cooperation outweigh those gained by excluding that competition.

The existence of a complex aggressive response directed against all non-clonemate conspecifics implies that intraspecific competition is quite costly for *Anthopleura elegantissima,* while the existence of the large clonal aggregations (Fig. 1) so common for this species and the specialization among individuals in these groups (reported here) imply significant advantage in cooperation under some circumstances. Probable advantages of living in aggregations as compared with solitary living include reduction of the effective surface area resulting in reduced water loss (Roberts, 1941) and reduced drag (M. Koehl, Duke University, personal communication), cooperation in holding larger food items, and physical exclusion of interspecific and non-clonemate intraspecific competitors. Although the overlap in requirements and capabilities among clonemates is obviously maximal, more is to be gained by cooperation with them than with any other genotype. Since members of an aggregation are genetically identical, an advantage gained by an anemone for any group member is an advantage to its own genotype; and pure altruism will have the same evolutionary effects as pure selfishness.

Animals that cannot distinguish near relatives from other conspecifics must choose between the benefits of interference and those of cooperation. The clonal aggregating form of *Anthopleura elegantissima* can benefit maximally from both types of interaction only because the individuals can distinguish clonemates from all other members of the species, (Francis, 1973b).

I argue first that intraspecific competition for space will be important for an animal like *Anthopleura elegantissima,* and further that the existence of the intraspecific aggressive behavior and the specialized equipment involved in intraspecific aggression is evidence that such competition actually is important. This leaves an implied question unanswered: what, if anything, prevents intense and wasteful competition among clonemates?

Colonial organisms (*i.e.,* organisms that maintain connections among clone members) show coordinated and orderly growth and division resulting in regular spacing of the individuals and expansion of the colony. This is only possible because the resources of a colony can be transported to budding areas.

Binary fission in *Anthopleura elegantissima* produces separate, slow-moving individuals rather than an orderly array of interconnected individuals. Consequently, growth and asexual reproduction are not easily coordinated to accomplish orderly extension of the group as they are in colonies.

Over-crowding resulting in competition for space and/or food will be a problem within dense aggregations of any sessile organism if the individuals continue to grow independently. The anemones will continue to grow if food is available in excess of that required for maintenance and activities unless they stop feeding or use the energy to produce more gametes. It seems unlikely that the anemones will stop feeding when crowded; however, gonads have been observed to develop more than once a year (B. Jennison, University of California, Berkeley, personal communication). Since smaller animals have larger surface to volume ratios than larger animals of the same proportions, asexual reproduction among anemones in a crowded area will probably increase the combined pedal disc area and thereby worsen any crowding problem. It is not yet clear what factors control growth, fission or sexual reproduction.

Fredericks (1976) suggests that movement in response to oxygen gradients can effect spacing within aggregations of *Anthopleura elegantissima*. Nonetheless, a clonal group that continues to expand will eventually occupy all the suitable substratum available in a particular location and crowding will occur. When subject to adverse conditions in the laboratory, these anemones can detach and rapidly re-attach (personal observation; and K. Sebens, University of Washington, Seattle, personal communication). In the field I have occasionally found unattached individuals in tightly crowded tidepools. Detachment could serve as a dispersal mechanism for clones that have saturated the holding capacity of their immediate surroundings.

How might morphological specialization arise within a clonal aggregation? Barring somatic mutation, the individuals are genetically identical and of the same age, having arisen from the same zygote. Variation among clone members is possible only to the extent that genetic expression can be affected by the environment.

Within the phylum Coelenterata are species showing all degrees of colony organization and individual specialization. At one extreme, the Siphonophora have been described as super-organisms because the interconnected and highly polymorphic polyps are organized into well coordinated colonies with distinctive species-specific morphologies. At the other extreme, the sea anemones (Actiniaria) do not remain attached and until now were not known to show specialization or group organization.

The organization within clones of *Anthopleura elegantissima* described here is unique for the phylum, in being social (*i.e.*, involving separate individuals) rather than colonial (involving attached individuals). Ability to specialize and to coordinate is limited by lack of connection among individuals. Without gut connections between clonemates, each anemone must feed for itself. Without nervous, neuroidal or circulatory connections with clonemates, and lacking a CNS and sophisticated light or sound receptors, each anemone must respond individually to its immediate surroundings. Any coordination of activity must involve either pheromones (Howe, 1975) or direct contact.

There are two different, although not mutually exclusive models that might explain the nonrandom arrangement of somewhat specialized individuals present in the clonal aggregations: *model one*—individual development during growth and regeneration might be affected directly by differences in microhabitat; and *model two*—unequal divisions and imperfect regeneration could produce variation within a clone, and individual success might then depend on microhabitat.

The data for sexual reproduction in relation to clonal position (Fig. 5) are most easily interpreted using the first model. The specific hypothesis I am presently testing is that the interclonal border warriors use enough energy in intraspecific aggression that they lack energy reserves for gamete production.

The lower fissioning rates of the warriors as compared with free edge or midclone animals (Table III) is probably also a result of their experience at the interclonal border (model one) rather than a variation favored by conditions at that location (model two). If there is insufficient energy for sexual reproduction among these animals, it is reasonable that growth rates and possibly also fission rates might be reduced. Coordination of growth rate and fission rate is necessary for control of individual size. Although it is not yet clear what factors are involved in triggering fission, the relationship is certainly not simply that animals reaching a certain size in each clone divide. The data given above for daughter pairs produced by fission include wet weights for four animals from the same clone (animals 1, 2, 3, and 8). Variation in size at the time of fission is considerable; wet weights of the pre-fission anemones were 1.70 g, 0.72 g, 0.59 g and 0.57 g, respectively.

There could be unequal success among animals of different sizes in particular locations. Fission alone will produce size variation within clones unless growth and division are synchronous. The range of such variation will be increased by inequalities in fission (documented here) and by shrinking of starved animals (Muscatine, 1961). In the crowded centers of aggregations, large animals might over-reach smaller clonemates and get more food, causing the larger anemones to get larger and the smaller ones to get smaller. At the edges of the clones, however, being small might not be such a disadvantage since the tentacles on one side of even small anemones will extend beyond the clone.

The acrorhagi of a given individual are usually asymmetrical in their arrangement (personal observation) and variable in size (Fig. 4, data collected separately for each individual, but grouped here); furthermore there is no apparent consistency among clonemates in their patterns of acrorhagus arrangement. Since fission is often unequal, this and the variation in acrorhagus number might be explained by failure of the anemones to replace some or all of the acrorhagi lost during fission. This explanation probably accounts for the variation in acrorhagus number among individuals from the middle of clones and from isolated clones (Table II).

Might position-related differences in acrorhagus size and number be a matter of differential success of diverse morphological types arising randomly in this way;

that is, can this situation be described using model two? If interclonal border anemones have more and larger acrorhagi only because anemones with little weaponry cannot long survive at that location, the variance for both acrorhagus size and the number of acrorhagi per individual should be reduced at that location. The variances are consistently higher, not lower, among the warriors than among their clonemates (Table II and Fig. 3). Furthermore, the sample means for the warriors are at the extremes of variation for the other clone members sampled (*c.f.*, Fig. 3 and Table II; Fig. 4 and the data in the corresponding text). To explain these data using only model two it is necessary to postulate not only selection at the interclonal borders favoring the warrior morphology, but also selection at midclone and free edges favoring animals with reduced weaponry. These data are perhaps more easily interpreted using model one or a combination of the two models. The hypothesis presently being tested is that anemones at the interclonal borders produce more and larger acrorhagi in response to their experience there.

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SUMMARY

The aggregating form of the anemone *Anthopleura elegantissima* often lives in closely packed groups composed of genetically identical individuals. These clonal aggregations remain separated from each other because contact between non-clonemates elicits intraspecific aggression (Francis, 1973a & b).

Since the anemones live in a dependable environment, are long-lived, have indeterminate growth, and reproduce sexually and asexually, and since predation does not appear to be severe, intraspecific competition for space is quite important. The ability to distinguish clonemates from non-clonemates allows the anemones to benefit from group living while interfering with all other conspecific competitors. A high proportion of the costs of this intraspecific aggression is paid by the anemones living at interclonal borders.

The interclonal border warriors have more and larger acrorhagi (specialized structures used in intraspecific aggression) than clonemates elsewhere in the aggregation and were without gonads in June when many of their clonemates were ripe. They are smaller on an average than midclone anemones but not smaller than clonemates from an edge of the aggregation remote from other clones. In the field, the fission rates of warrior anemones is not higher, and may be lower than that of clonemates in other parts of the same aggregation. Lack of gonads, small size, and low fission rates probably indicate that the warriors have less energy available for growth and reproduction than do their clonemates away from the battle zone.

Without physical connections among the individuals, the ability to coordinate and communicate is limited. Each anemone responds to its particular circum-

stances, and in so doing may fortuitously benefit its clonemates. Natural selection acts on the genotype (the clone). This is a very simple form of social organization both functionally and evolutionarily.

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STUDIES ON THE SIZES, SHAPES, AND THE DEVELOPMENT OF THE LORICA OF AGGLUTINATED TINTINNIDA[1]

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The ciliates, Tintinnida, are well known to be capable of accumulating particulate substances on their loricae. Gold and Morales (1976), using scanning electron microscopy, demonstrated that there were obvious differences in the nature of the particles that were utilized by members of the genera *Stenosemella and Tintinnopsis.* The former produced loricae that were distinctly arenaceous and composed principally of mineral particles of nonbiological origin; *Tintinnopsis tubulosoides* appeared to be agglutinated with nonbiogenic grains and flakes, but also with numerous fragments of biogenic material such as protozoan shells, coccoliths and diatom frustules. The significance of the differences in the materials utilized by species of the two genera, was suggested to lie in developmental processes and in the location where the loricae were produced, *e.g.,* the sediments or the water column. It followed from that hypothesis that the types of materials utilized should be of evolutionary and taxonomic importance. From another point of view, the agglutinating species have the potential of concentrating insoluble minerals directly, or soluble forms sorbed to silt-sized particles. This route may be one in which minerals are moved along the food chain to humans, hence there is a practical need to identify the substances that are commonly utilized by agglutinating Tintinnida in diverse habitats.

This communication deals with three aspects of tintinnid biology concerning the shapes, sizes, and the development of the loricae. First, with the aid of scanning electron microscopy, the agglutinated species of the Woods Hole, Massachusetts region are characterized according to the nature of the particles accumulated and the apparent methods of lorica construction. Second, daily differences are reported in the mean length of the loricae in populations of *Tintinnopsis acuminata.* Third, varieties and atypical loricae of the same species are identified for some common forms.

The following terminology is used here to describe particle accumulation in the lorica development process: *agglutination* refers to the process of accumulation of particles regardless of the source and types of materials utilized. *Arenaceous* refers to the appearance of a lorica that was produced predominantly of mineral grains. The grains appear to be cemented together by what may be an active process; such forms could have an underlying organic matrix present. A lorica is considered to be agglomerated if it has both mineral grains and a large proportion of fragments of biological origin adhering to it. Agglomerated loricae differ from the arenaceous types in the amount of biogenic material present and in their

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TABLE I

Species of Tintinnida of the Woods Hole region. June–August, 1974–1975.
The numerically important species during 1975 are indicated by an asterisk.

- *Family Tintinnididae*
    - *Tintinnidium fluviatile**
- *Family Codonellidae*
    - *Tintinnopsis acuminata**
    - *T. baltica*
    - *T. dadayi**
    - *T. levigata**
    - *T. minuta**
    - *T. nucula*
    - *T. platensis**
    - *T. radix*
    - *T. rapa**
    - *T. sufflata**
    - *T. vasculum*
- *Family Codonellopsidae*
    - *Stenosemella oliva**
    - *S. ventricosa**
- *Family Coxliellidae*
    - *Helicostomella subulata*
    - *Metacylis* (2 species) (Figure 3m)*
- *Family Ptychocylididae*
    - *Favella ehrenbergii*
- *Family Tintinnidae*
    - *Tintinnus angustatus**
    - *T. pectinis**

appearance—the particles on the loricae of agglomerated species seem to have been taken up randomly.

MATERIALS AND METHODS

Specimens were collected by hand-towing a twelve inch diameter plankton net (mesh size 20 μm) from the supply dock at the Marine Biological Laboratory, Woods Hole, Massachusetts (Eel Pond), and from the pump pier extending into Great Harbor. Samples used to identify species and for measurements were preserved either in Schaudinn's solution or ~ 10% formaldehyde in sea water; specimens prepared for scanning electron microscopy (SEM) were generally selected from the samples live and micropipetted into a suitable killing agent such as Schaudinn's solution or Karnovsky's fixative (1965).

Measurements of loricae were made on wet specimens contained in a Sedgewick-Rafter counting chamber using a Wild M-20 microscope equipped with an ocular micrometer.

Specimens to be examined by SEM were thoroughly rinsed in glass distilled water, by micropipetting the cells through at least five 1 ml changes of water. Rinsed specimens were usually dried in air on cover glasses or in a Sorvall critical point drying system. Specimens were either left on the cover glasses or placed on double coated Scotch tape, attached to a specimen mount, and then coated with 200 Å of gold/palladium alloy in a Tousimis Samsputterer. Finally, specimens were examined and photographed in a JEOL JSM-35 scanning electron microscope.

Loricae that were used in electron probe analysis were from formaldehyde-killed cells that had been thoroughly rinsed in distilled water before being mounted on spectroscopically pure carbon specimen mounts. Analysis for minerals was then performed in a JEOL JSM-U3 scanning electron microscope equipped with an Ortec energy dispersive probe microanalyzer.

RESULTS

The species of Tintinnida that were collected in the Woods Hole, Massachusetts region during June to August 1974–1975 are listed in Table I. The table includes all of the species encountered. With the exception of *Favella ehrenbergii,* all of the species found in 1974 were present in 1975; seven additional species not detected in 1974 were present in 1975 (Gold, 1974; Gold and Morales, 1975b).

A greater variety of species was found in Great Harbor, considered here to be a neritic well-mixed environment, compared with Eel Pond—a relatively protected habitat. When the species estimated to be numerically important in both habitats were plotted according to the day of their occurrence, the pattern was a recurring one for most of the species, indicating the presence of endemic populations in these waters at that time of the year. Some species were conspicuous by the absence of a recurring pattern. In Eel Pond, for example, a *T. dadayi* population appeared suddenly, remained for approximately two weeks, then suddenly disappeared. A similar pattern was seen for *Metacylis* sp. and *Tintinnus angustatus* in Great Harbor. Abrupt disappearances of populations are recorded here for *T. rapa* in Eel Pond and for *T. sufflata* and *T. levigata* in Great Harbor. The occurrence of species seemed unrelated to water temperature in Eel Pond (Fig. 1).

A daily difference was found in the mean length of *T. acuminata* loricae in Eel Pond. Figure 2 shows a comparison of the mean length of the lorica and the standard error of the mean during periods of relatively high abundance in 1974 and 1975. The data were plotted with reference to the day on which the maximum

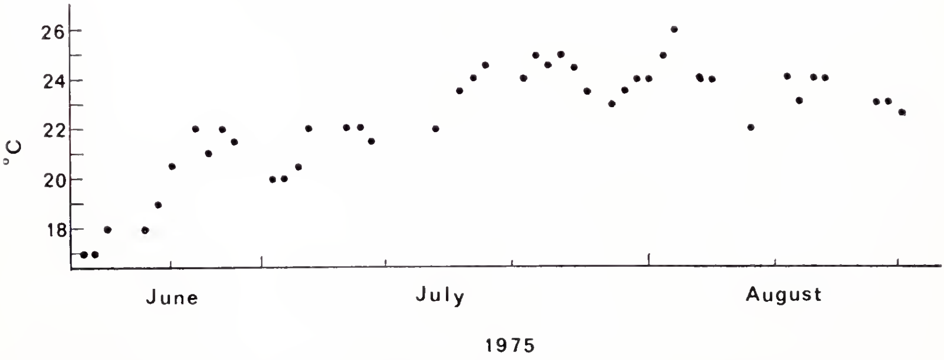


FIGURE 1. Water temperature at the surface in Eel Pond, 1975.

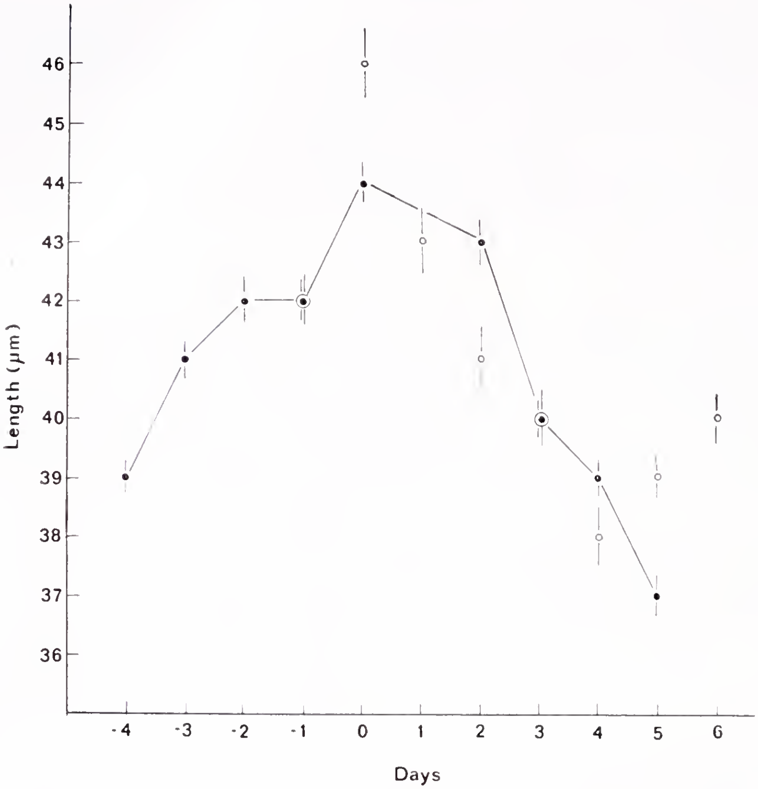


FIGURE 2. Daily mean length and the standard error for two populations of *T. acuminata*. Open circles indicate 1974; closed circles, 1975.

mean length was detected (day zero) so that the shapes of the curves could be compared quickly. The day-to-day differences were clear-cut, thus obviating the need to subject the dimensions to statistical analysis for significance.

Polymorphic varieties of loricae were detected in the plankton (Fig. 3). Besides these polymorphic varieties, atypical forms of one species were found, which are interpreted as developmental stages in lorica building. The normally developed, mature form of the lorica of *T. dadayi* is shown in Figure 8; Figure 9 is an advanced two-flare stage. Specimens such as the latter, as well as others with tubular extensions and three flares, were present in large enough numbers to warrant their being viewed as normal constituents of the plankton. Specimens were also found that lacked a flare; these were probably immature forms and accounted for a maximum of 12% of one *T. dadayi* population.

The pertinent dimensions used to identify the Woods Hole Tintinnida follow. The principal dimension used to identify forms of a lorica that were similar was the diameter of the cylindrical portion of the bowl. This dimension was found previously to be a very stable feature of the lorica structure (Gold and Morales,

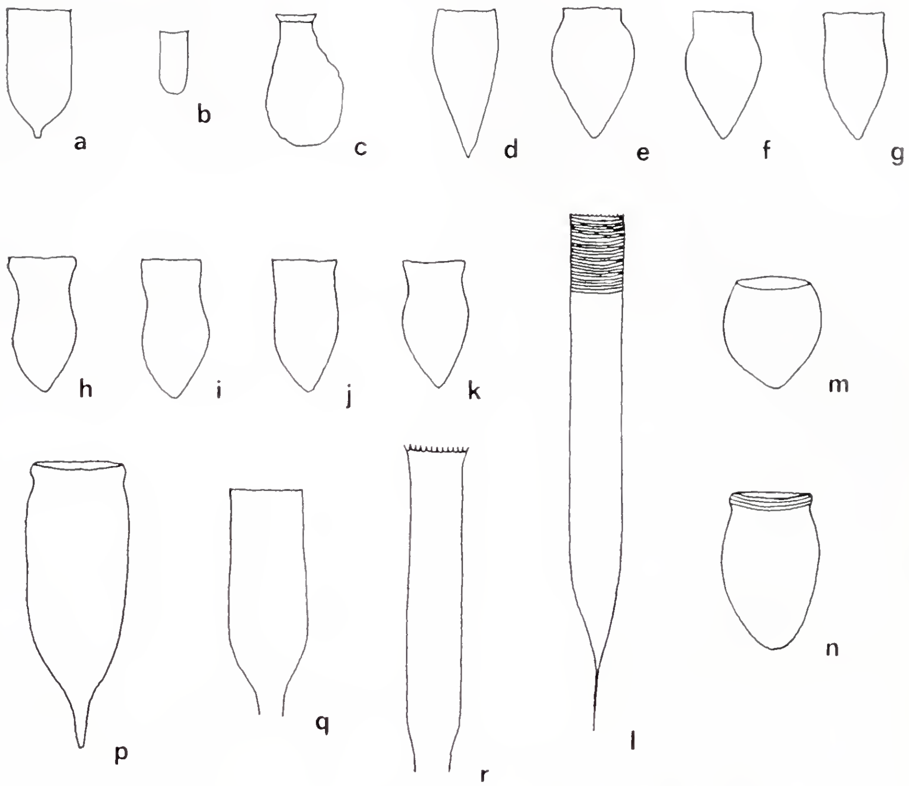
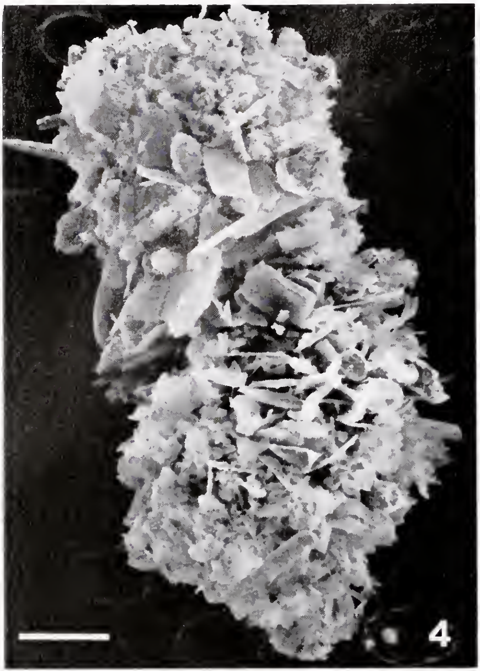


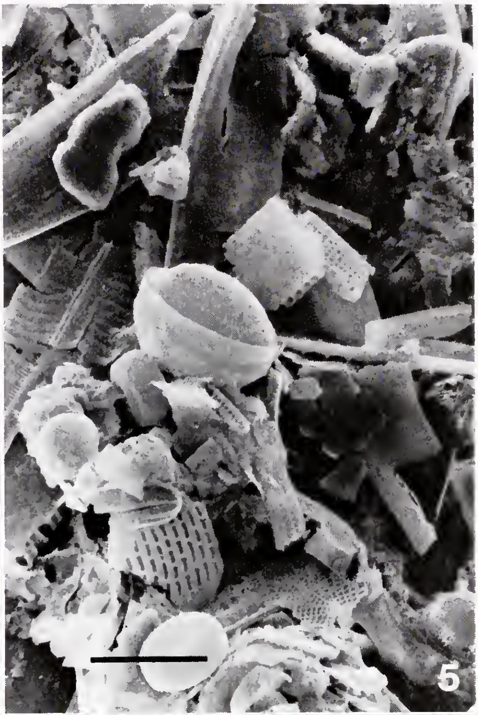
FIGURE 3. Varieties of loricae and those not viewed by SEM. Magnification is 400×, except for *Favella ehrenbergii* (p) 200×. (a) *Tintinnopsis levigata,* (b) *T. minuta,* (c) *T. nucula,* (d–g) *T. rapa,* (h–k) *T. sufflata,* (l) *Helicostomella subulata,* (m) *Metacylis* sp., (n) *Metacylis* sp., (p) *Favella ehrenbergii,* (q) *Tintinnus angustatus,* (r) *T. pectinis.*

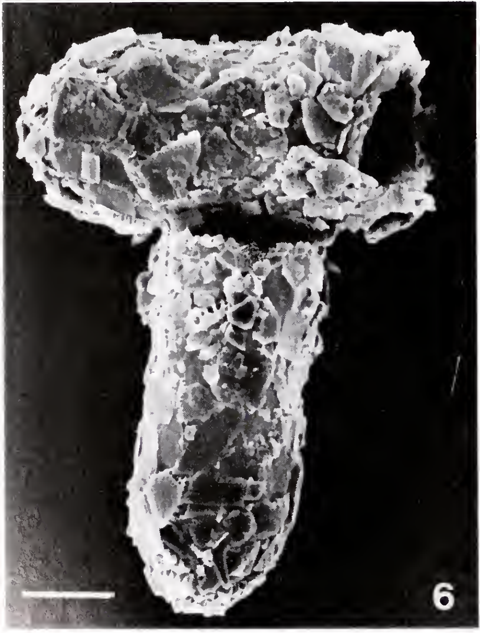
1975c); the length, on the other hand, often varied considerably, so a range is given for this dimension.

The sizes of the particles that were accumulated by agglutinating species, as well as the major categories of materials utilized (biogenic or nonbiogenic), are summarized below. It is noteworthy that the particles surrounding the oral aperture of certain species loricae were smaller than the grains found elsewhere on the lorica. The arenaceous *Tintinnopsis* and *Stenosemella ventricosa* in particular, showed selectivity for particle sizes rather clearly. The range of particle sizes around the neck of *T. rapa* and *S. ventricosa* was less than 1–4 μm, in contrast to particles up to 8 and 14 μm, respectively, elsewhere on the lorica.

Three representative particles attached to arenaceous loricae of *S. ventricosa* and *Tintinnopsis* sp. were subjected to electron probe analysis. The principal peak resulted from silicon with very minor amounts of calcium and potassium present, these being almost undetectable in one analysis. Thus we consider the principal grain type to be quartz.







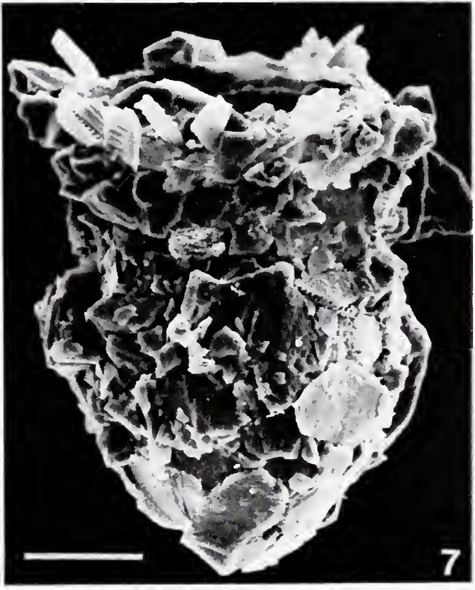


FIGURE 4. Lorica of *Tintinnidium fluviatile*. Desiccation was by the CO_2 critical point drying method to retain the tubular structure. Scale bar equals 15 μm.

FIGURE 5. Portion of an air-dried lorica of *T. fluviatile*. Scale bar equals 7.5 μm.

FIGURE 6. Loricae of *Tintinnopsis acuminata*. Scale bar equals 10 μm.

FIGURE 7. Lorica of *T. baltica*. Scale bar equals 10 μm.

The revised taxonomic scheme of Corliss (1961) is used below in listing the families of Tintinnida of the Woods Hole region. The length of the lorica in μm is denoted by the letter *L*; *W*, the width in μm; and *n*, the sample size.

TAXONOMY

Family Tintinnididae

Tintinnidium fluviatile (Stein) Kent. Figures 4 and 5.

L = 54–164 (*n* = 237) ; W = 48 ± 5 (*n* = 219). See Fauré-Fremiet, 1924; Kofoid and Campbell, 1929; Silva 1952; Gold and Morales, 1975c. Species agglomerated with particles that are predominantly biogenic in origin; whole diatom frustules may be incorporated. Range of particle sizes utilized less than 2.5–36 μm.

Family Codonellidae

Tintinnopsis acuminata Daday. Figure 6.

L = 31–59 (*n* = 2,115) ; W = 20 ± 1 (*n* = 202). See Kofoid and Campbell, 1929; Marshall, 1969; Gold and Morales, 1975c. Species agglomerated with particles that are mostly nonbiogenic flakes. Biogenic particles include fragments of diatom frustules, etc. Particles surrounding the rim of the lorica are smaller than on the bowl, *e.g.* rim, less than 1–2.5 μm; posteriorly, less than 2–8.5 μm.

T. baltica Brandt. Figure 7.

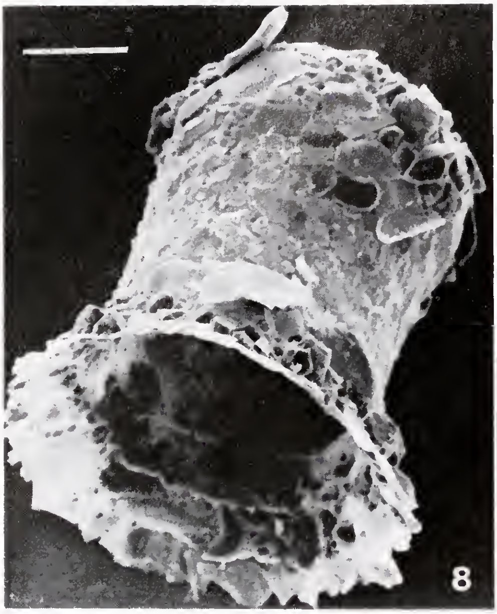
L = 53–67 (*n* = 19) ; W = 40 ± 4 (*n* = 19). See Kofoid and Campbell, 1929; Hada, 1937; Balech, 1945, 1948; Silva, 1950; Gold and Morales, 1975c. Species agglomerated with a large proportion of particles of nonbiogenic origin, but also with fragments of diatom frustules (less than 1–6 up to 10 μm).

T. dadayi Kofoid. Figures 8 and 9.

Single flare specimens only: L = 62–81 (*n* = 162) ; W = 43 ± 2 (*n* = 162). See Kofoid and Campbell, 1929; Durán, 1957; Gold and Morales, 1975c. Species agglomerated with a large proportion of flakes of nonbiogenic origin, but also with considerable biogenic material present including fragments of diatom frustules. Range of particle sizes utilized less than 2–10, up to 15 μm.

T. levigata Kofoid and Campbell. Figure 3a.

L = 41–57 (*n* = 26) ; W = 22 ± 1 (*n* = 26). See Kofoid and Campbell, 1929; Silva; 1950; Durán, 1965; Marshall, 1969; Gold and Morales, 1975b, 1975c. Referred to as *T. strigosa* (Gold and Morales, 1975b). Not viewed



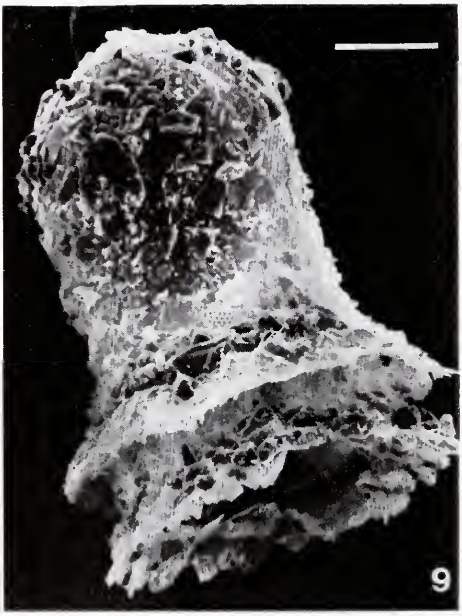


FIGURE 8. Lorica of *T. dadayi* showing spiral structure. Scale bar equals 15 μm.

FIGURE 9. Lorica of *T. dadayi* believed to be an advanced developmental stage produced by the posterior product of division following fission. Scale bar equals 17 μm.

in SEM. Lorica appears heavily agglomerated in light microscope. Posterior horn is closed in this variety.

T. minuta Wailes. Figure 3b.

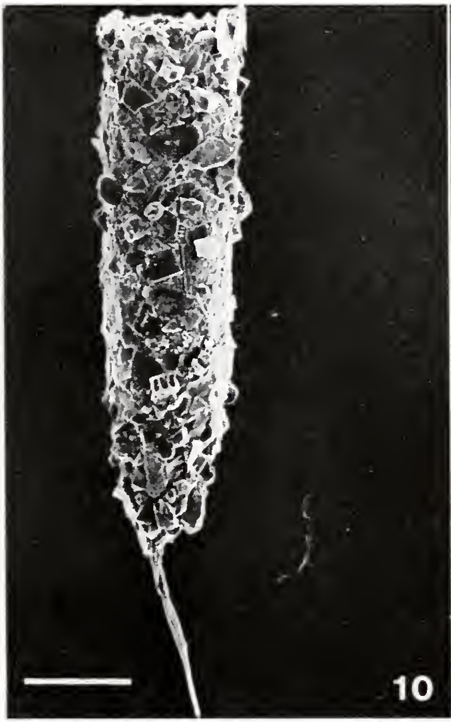
L = 18–34 ($n = 87$); W = 13 ± 1 ($n = 71$). See Kofoid and Campbell, 1929; Hada, 1938; Wailes, 1943; Marshall, 1969; Gold and Morales, 1975c. Also referred to as *T. nana* (Hada, 1938). Not viewed by SEM; loricae appear lightly agglomerated in light microscope.

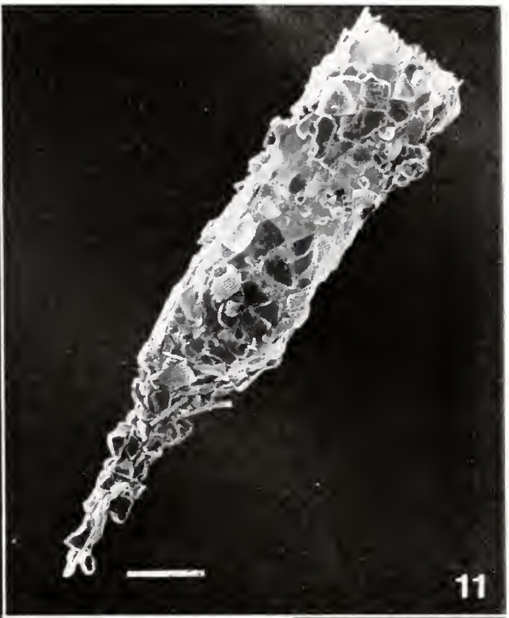
T. nucula (Fol) Brandt. Figure 3c.

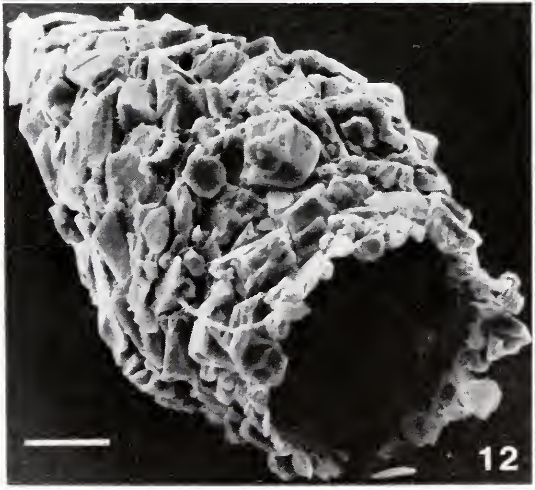
L = 46; W = 27; oral diameter = 15 ($n = 1$). See Kofoid and Campbell, 1929; Hada, 1938; Silva, 1954. Not viewed by SEM. Lorica appeared heavily agglomerated in light microscope. Though sample size was small, the specimen is included here because it is an unusual and distinctive one.

T. platensis Cunha and Fonseca. Figure 10.

Bowl L = 80–223 ($n = 82$); horn L = 35–76 ($n = 82$); W = 43 ± 2 ($n = 82$). See Cunha and Fonseca, 1912; Kofoid and Campbell, 1929; Hada, 1938; Cosper, 1972; Gold and Morales, 1975c. Referred to as *T.*







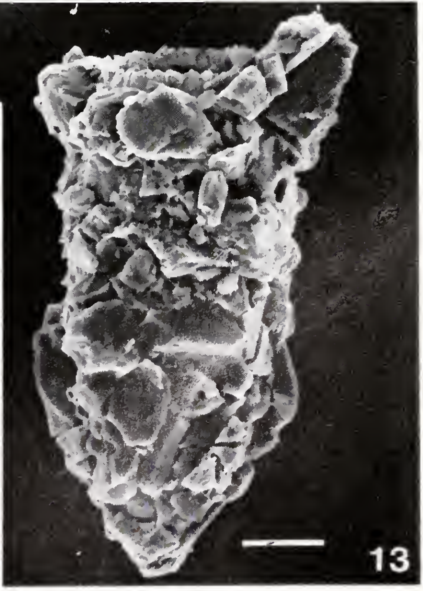
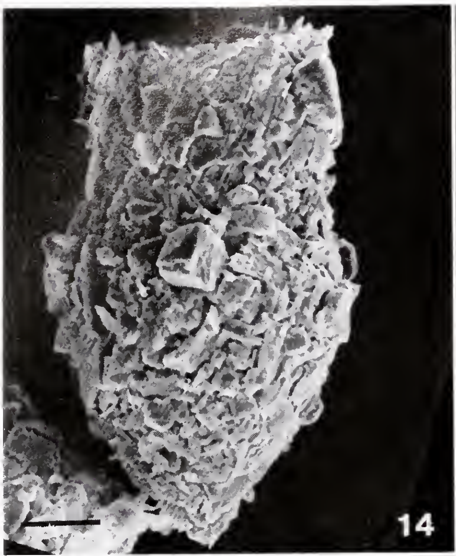
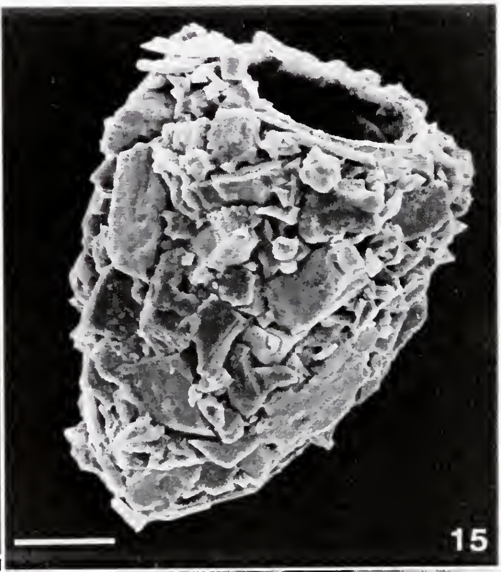
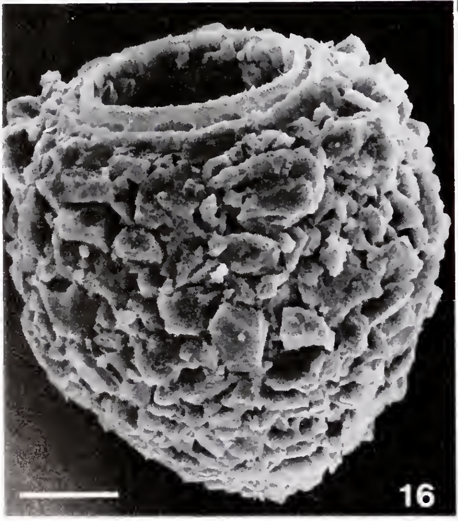


Figure 10. Lorica of *T. platensis*. Scale bar equals 32.5 μm.
Figure 11. Lorica of *T. radix*. Scale bar equals 20 μm.
Figure 12. Lorica of *T. rapa*. Scale bar equals 6 μm.
Figure 13. Lorica of *T. sufflata*. Scale bar equals 7 μm.

davidoffi (Calkins, 1902); renamed *Stylicauda platensis* (Balech, 1951). Species agglomerated with both biogenic and nonbiogenic particles, with flakes being the predominant nonbiogenic variety. Range of particle sizes utilized less than 2–13 up to 25 μm. Horn entirely unagglomerated and







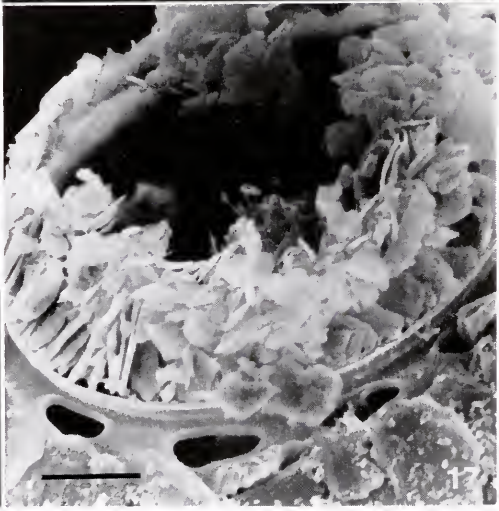


FIGURE 14. Lorica of *T. vasculum*. Scale bar equals 10 μm.
FIGURE 15. Lorica of *Stenosemella oliva*. Scale bar equals 11 μm.
FIGURE 16. Lorica of *S. ventricosa*. Scale bar equals 16 μm.
FIGURE 17. Anterior region of an *S. ventricosa* lorica showing fenestrae, smaller particles, and in the center, cilia of the contracted cell within. Scale bar equals 6.5 μm.

hyaline. No spiral structure evident as reported by Balech (1951) and Cosper (1972).

T. radix (Imhof) Brandt. Figure 11.

L (includes horn) = 114–198 ($n = 10$); W = 36 ± 1 ($n = 10$). See Hada, 1937, 1938; Balech, 1945; Cosper, 1972. Agglomerated with nonbiogenic particles, mostly flakes but also some grains. Biogenic particles also present. Great variability in length of horn (possibly broken during collection) gives extensive length range.

T. rapa Meunier. Figures 3d–g and 12.

L = 39–58 ($n = 103$); W = 23 ± 2 ($n = 92$). Variety (Figure 3e): L = 38–56 ($n = 28$); W = 28 ± 2 ($n = 28$); oral diameter = 22 ± 2 ($n = 28$). See Kofoid and Campbell, 1929; Hada 1937; Gaarder, 1946; Silva, 1952; Marshall, 1969; Gold and Morales, 1975c. Lorica has an arenaceous appearance due to the high proportion of nonbiogenic particles (mostly grains, but some flakes). Anterior rim consists of small particles (less than 1–4 μm); larger particles elsewhere on bowl (less than 1.5–8 up to 21 μm). Much polymorphic variability evident in Woods Hole specimens (see Figure 3). Confirms Hada's (1937) observation, namely, much variability in size and in length of the aboral conical region.

T. sufflata Hada. Figures 3h–k and 13.

L = 40–64 ($n = 121$); W = 22 ± 2 ($n = 121$). See Hada (1937) which shows specimens with an aboral aperture. The form of the lorica is similar to *T. rapa*; differs, however, in presence of constriction in middle of lorica and very slight widening anteriorly. Both species loricae constructed primarily with nonbiogenic grains. A rim of small particles has been found at the anterior opening.

T. vasculum Meunier. Figure 14.

L = 76–82 ($n = 5$); W = 49 ± 1 ($n = 4$); oral diameter = 39 ± 3 ($n = 5$). See Silva, 1954; Marshall, 1969; Gold and Morales, 1975c. Lorica has an arenaceous appearance due to the almost exclusive use of nonbiogenic grains of uniform size. The particles are presumed to be quartz. Particles are small for a lorica of this size (less than 1.5–6 up to 10 μm).

Family Codonellopsidae

Stenosemella oliva Meunier. Figure 15.

Bowl L = 48–68 ($n = 436$); W = 39 ± 2 ($n = 361$); collar = 25 ± 3 ($n = 166$). See Kofoid and Campbell, 1929; Gaarder, 1946; Durán, 1965; Marshall, 1969; Gold and Morales, 1975c. Lorica has an arenaceous appearance due to the high proportion of particles of nonbiogenic origin. Grain sizes are less than 1.5–13 up to 16 μm. The oval fenestrae present in the hyaline collar are often obscured by small flakes of nonbiogenic material that adhere.

S. ventricosa Claparède and Lachmann. Figures 16 and 17.

Bowl L = 72–86 ($n = 80$); W = 72 ± 3 ($n = 80$); collar = 41 ± 3 ($n = 77$). See Kofoid and Campbell, 1929; Gaarder, 1946; Silva, 1950; Durán, 1957; Balech, 1959; Marshall, 1969; Gold and Morales, 1975c. Lorica has an arenaceous appearance due to the high proportion of particles of nonbiogenic origin. The range of sizes of grains on the bowl was less than 2–14 up to 25 μm. Flakes usually present around the hyaline collar (sizes less than 1–3.5 μm); often obscure it and hide the oval fenestrae. Representative particles on the bowl were analyzed by electron probe and were determined to be quartz.

Family Coxliellidae

Helicostomella subulata Ehrenberg. Figure 31.

L = 134–242 ($n = 4$); W = 20 ± 1.5 ($n = 4$). See Kofoid and Campbell, 1929; Hada, 1937; Wailes, 1943; Marshall, 1969; Gold and Morales, 1975c. Lorica hyaline, narrow and cylindrical; tapers aborally to form a long, pointed horn. Anterior rim serrated; spiral structure prominent.

Metacylis sp. Figure 3m.

L = 44–56 ($n = 14$); W = 40 ± 2 ($n = 11$); oral diameter = 32 ± 1 ($n = 14$). Lorica hyaline without collar; widest in middle, narrows posteriorly forming a blunt point. Appears to be a new species.

Metacylis sp. Figure 3n.

L = 57; W = 40; oral diameter = 33 ($n = 1$). See Cosper, 1972. Lorica hyaline. Rim with 2–3 rings; indents below rim forming elongated rounded bowl.

Family Ptychocylididae

Favella ehrenbergii Chaparède and Lachmann. Figure 3p.

L = 159–218 ($n = 35$); W = 70 ± 2 ($n = 35$). See Kofoid and Campbell, 1929; Marshall, 1969; Gold and Morales, 1975c. Conspicuous by its absence from the plankton in 1975; dimensions given are from 1974. Large hyaline lorica with short aboral horn present. Constriction present immediately below anterior rim. Irregular polygonal structure visible at 1000 ×.

Family Tintinnidae

Tintinnus angustatus Daday. Figure 3q.

L = 93–118 ($n = 15$); W = 35 ± 2 ($n = 15$); aboral opening = 14 ± 2 ($n = 15$). See Kofoid and Campbell, 1929; Hada, 1938. Lorica hyaline, elongate, slightly expanding posteriorly, then narrows to aboral opening.

T. pectinis Kofoid and Campbell. Figure 3r.
L = 125–171 ($n = 69$); W = 22 ± 1 ($n = 69$). See Kofoid and Campbell, 1929; Wailes, 1943. Lorica hyaline, slightly tapered posteriorly. Anterior rim serrated; open aborally.

DISCUSSION

Some of the methods suggested to account for lorica building by tintinnids were summarized by Tappan and Loeblich (1968). None of the hypotheses took into account the possibility that construction could take place in the sediments or at the sediment-water interface. The attractiveness of this hypothesis, suggested first by Gold and Morales (1975b, 1976), is that these locations would provide easy access to the materials that seem to be actively selected for lorica building by certain species. In these studies, four distinctly different processes of lorica building were discerned by SEM; they are, from all appearances, significant at the generic level. The following are cited as representatives of the different developmental processes: *Tintinnidium fluviatile* (Fig. 4); *Tintinnopsis acuminata* (Figure 6); *Tintinnopsis rapa* (Fig. 12); and *Stenosemella ventricosa* (Fig. 16).

Loricae of *Tintinnidium fluviatile* appear to be relatively soft in consistency. Unless the cell has been fixed quickly and is retained within the lorica, the structure can easily go unnoticed in a preserved sample due to its resemblance to a clump of detritus. The specimen shown in Figure 4 was removed from the plankton while the organism was swimming and then placed in the preservative. This assured that the structures photographed were loricae and not detritus. The adhering particles are extremely varied and large by comparison with the sizes of particles on other species loricae. It is noteworthy that this was the only species where entire diatom frustules were found adhering to the lorica. The appearance of the lorica and the apparent indiscriminate uptake of particles, indicates that particle accumulation by this species is random and perhaps a passive process by comparison with the methods discussed below.

Various *Tintinnopsis* species have been grown *in vitro* (*e.g.*, Gold, 1968, 1973); in the absence of added particles, each has been found to produce a clear, organic lorica. Presumably, a similar structure lies beneath the particles on the *T. acuminata* lorica and on other *Tintinnopsis* that have a similar agglomerated appearance, *e.g.*, *T. platensis*, *T. dadayi*, *T. baltica*. A spiral structure is visible on the *T. dadayi* lorica shown in Figure 8. It can be assumed from the appearance of these loricae, and it was also demonstrated experimentally by the addition of small particles to cultures, that the organic matrix of the lorica is adhesive or is coated with an adhesive substance. *Tintinnopsis* spp. have specialized cilia used in molding the anterior lip of the lorica (Gold, 1968); manipulation by these membranelles, along with passive accumulation by adhesion, seem to be the methods of particle accumulation used by this group of organisms.

The designation *Tintinnopsis* is used tentatively for *T. rapa.* The lorica construction is obviously different from that of *T. acuminata* described previously. Nevertheless, using accepted taxonomic criteria, namely size and the shape of the lorica, this specimen is deemed to be correctly identified. The particles utilized by this species appear to be almost exclusively quartz. Particle selectivity is strongly suggested by the appearance, but selection for the habitat in which the lorica is produced is an alternative explanation for the species using only quartz particles. The presence of particles at the rim that are smaller than elsewhere on the lorica also suggests selectivity, but this too could be the result of selecting a certain habitat for lengthening the lorica. These loricae are arenaceous, by the definition given previously, and may or may not have an organic lining beneath the particles. It seems more likely, however, that the particles are simply cemented together to form the bowl, and that extensions are made possible by an adhesive being applied at the rim and the organism darting to the sediments where it encounters minute quartz particles. Since the accumulation of particles by this species demonstrates some selectivity, particle uptake by organisms such as *T. rapa* is considered to be an active process.

There are similarities seen in the construction method used by *Stenosemella ventricosa* and the method used by *T. rapa.* In both species, some selectivity has occurred for the types of particles utilized and for their sizes. The appearance of such loricae suggests that this species produces loricae in the sediments by first secreting an organic adhesive in close proximity to the cell membrane. Once the organic matrix has been coated with particles, the cell leaves the sediments and takes up its planktonic existence. These loricae are elongated throughout the life history of the cell, by adhesion of particles in the collar region; when abundant, they may obscure this hyaline structure.

The observed changes in the daily mean lorica lengths reported for *T. acuminata* are interpreted as being the result of cell division and lorica growth processes. We recognize that other possible causes exist that could account for the size differences, *e.g.*, genetic considerations, life history, food availability and its quality. The known fission and lorica building processes, however, seem more likely as explanations. The smallest loricae in the population are interpreted as immature forms, products of the anterior daughter cell following fission. The longest specimens are viewed as representatives of an older age class of loricae. Presumably the posterior daughter that retained the old lorica following cell division continued to add on substance in its normal process of elongation. The observations by Entz (1909), Nie (1933) and Gold (1974) where they suggested that the different sizes of loricae in a population were indicative of age, add support to this explanation.

In the past, a lorica such as the one shown for *T. dadayi* with two flares (Fig. 9), had been considered abnormally developed (Durán, 1957) or an attempt at lorica repair by the cell (Biernacka, 1965). In all likelihood, it is neither; it is viewed here as an entirely normal structure, the second flare being produced by the posterior daughter that had retained the old lorica at the previous fission. Should this be true, such distinctive forms would be extremely useful in determining the age classes of tintinnids in a plankton sample, in measuring the time between fissions *in situ*, and for determining the lifetime of a cell in the plankton in the presence of predators.

The factors that are responsible for the occurrences of various species throughout the summer months are obscure. Temperature in itself does not appear to be a major contributor to successions during the three months of observations. Dissipation of blooms of tintinnids could sometimes be linked to adverse weather conditions that produced turbulence; it is likely that intensive and variable predation were also major contributors to the disappearance of certain species. Studies are needed in protected tanks or pools to determine more precisely the causes of successions of populations of Tintinnida.

It is beyond the scope of this study to revise the taxonomy of the genus *Tintinnopsis*. It is impossible from these limited observations, to determine which developmental method—that leading to an arenaceous or to an agglomerated appearance—is the more primitive one. Clearly, though, future taxonomic revisions must take into consideration the nature of the particles utilized; more important, the incorporation process itself must be considered.

Summary

The Tintinnida of the Woods Hole, Massachusetts region were identified during two consecutive summers; loricae of eleven species of the agglutinated variety were studied by scanning electron microscopy. There were distinct differences in the types of particles utilized. In some species, quartz was incorporated almost to the exclusion of all other minerals. Biogenic particles such as fragments of diatom frustules, protozoan shells, and coccoliths were conspicuous on a number of species. The authors suggest that this observable difference in the nature of the agglutinated particles is significant at the generic level.

Variability in the sizes and shapes of the lorica of *Tintinnopsis acuminata* and *T. dadayi* are discussed in relation to cell growth and lorica developmental processes.

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CHELIPED LATERALITY IN *CALLINECTES SAPIDUS* (CRUSTACEA: PORTUNIDAE)

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In bilaterally symmetrical animals possessing paired appendages, the members of each pair are usually morphologically identical. This generalization holds true for crustaceans often postulated as being most similar in form to ancestral stocks. However, members of several reptantian decapod families (Nephropidae, Calappidae, Xanthidae, Portunidae, Gecarcinidae, Ocypodidae) possess dimorphic chelipeds in both sexes, each cheliped having a different function during feeding. Typically, one cheliped is larger and possesses large bluntly-tipped teeth (the major cheliped or crusher) and the other cheliped is smaller and possesses small sharply tipped teeth (the minor cheliped or cutter). Members of other reptantian families (*e.g.*, the Parthenopidae, Majidae) may also possess dimorphic chelipeds. However, it is unclear if these species possess true crusher and cutter chelipeds having different functions during feeding, and if the dimorphism exists in both sexes (see Vernet-Cornubert, 1957).

While cheliped laterality, or the location of the crusher and cutter chelipeds (sometimes called handedness), has been studied in the special case of male *Uca* spp. (Przibram, 1931; Vernberg and Costlow, 1966), laterality of reptantians having crusher and cutter chelipeds has rarely been examined. In a sample of 2430 *Homarus americanus,* Herrick (1909) found 1266 had a left crusher and right cutter, and 1164 had the opposite configuration. Although Herrick cites these results as evidence for a 1 : 1 ratio, we find his data significantly ($X^2 = 4.28$, $P < 0.05$) different from a 1 : 1 ratio. Przibram (1931) claimed that all Brachyura start out with a right crusher, and that reversal of this configuration in some crabs is caused by autotomy of the crusher. This conclusion was based on experiments with *Portunus corrugatus, P. depurator, Carcinus maenas,* and *Eriphia spinifrons.* However, subsequent researchers studying *Calappa* spp. (Shoup, 1968), *Portunus puber* (Ebling, Kitching, Muntz and Taylor, 1964) and *Carcinus maenas* (Seed, 1969) only state that "usually" or "normally" the crusher is on the right and the cutter is on the left. Schäfer (1954) illustrated the dimorphic chelipeds of many brachyurans, but did not state whether the cheliped configurations of the specimens illustrated were representative for the species. He did state that 8% of all *Portunus holsatus* lack the large tooth on the right cheliped, but was unclear whether this condition was due to excessive wearing of an otherwise normal crusher, or whether these crabs possessed two cutters. In 1601 *Neopanope texana sayi,* Swartz (1972) found that 79.9% had a right crusher (major cheliped) and left cutter (minor cheliped), 12.6% had the opposite configuration, 3.4% had two cutters and 4.0% were missing one or both chelipeds. He apparently did not consider a possible relationship between crab size and cheliped configuration, but did note that autotomy of the crusher did not cause reversal of the original con-

TABLE I

Collection data for the five Florida samples.

Sample location	Number	Collection date (1975)	Mean carapace length (±1 s.d.)
Gulf Coast			
St. Marks River (Wakulla County)	340	May 21	60.3 ± 5.80
Goose Creek Bay (Wakulla County)	181	May 26 to July 18	38.5 ± 8.70
Dickson Bay (Wakulla County)	274	August 4, 5	21.7 ± 8.04
Atlantic Coast			
Trout River (Duval County)	117	June 17	60.3 ± 5.54
Nassau Inlet (Duval County)	184	June 14	52.6 ± 5.95

figuration in 36 crabs maintained in the laboratory. The apparent rarity of specimens of *Calappa* with left crushers recently led to the erection of a new genus based on a single fossilized left crusher, which taxon was subsequently synonomized with *Calappa* after a more rigorous examination of the phenomenon of reversal (Lewis, 1969).

The apparent difference in cheliped laterality between macruran and brachyuran decapods was noted by Aristotle (quoted by Herrick, 1909), who wrote, "In the Caribi and in the Carcini the right claw is invariably the larger and stronger. In the Astaci alone it is a matter of chance which claw is the larger, and this in either sex."

Callinectes sapidus Rathbun possesses crusher and cutter chelipeds, with a large blunt tooth located proximally on the dactylus of the crusher of most individuals (see Williams, 1974, Fig. 16A). The chelipeds are used differently when feeding on the gastropod *Littorina irrorata* (Hamilton, 1976) and on the bivalve *Crassostrea virginica* (J. G. Halusky, unpublished observations). Williams (1974) states that the crusher and cutter of *Callinectes* are "usually" on the right and left, respectively, a few crabs have two cutters, and almost none have two crushers. However, he includes no quantification. Herein is presented laterality data based on six samples of *C. sapidus*, the possible sources of variation among samples are considered, and it is concluded that Przibram's (1931) aforementioned generalization holds for this brachyuran species.

MATERIALS AND METHODS

To quantify the incidence of crusher and cutter laterality in *Callinectes sapidus*, 1096 crabs from five different locations on the Gulf and Atlantic coasts of Florida, USA were examined, along with 60 crabs hand-reared at the Duke University Marine Laboratory, Beaufort, North Carolina. Table I summarizes collection data for the five Florida samples.

Crabs from the St. Marks River, Trout River and Nassau Inlet were examined in processing houses after being trapped commercially. Crabs from Goose Creek Bay were netted in the upper intertidal zone during high tide. Crabs from Dickson Bay were either netted from a dock or captured in a shrimp trawl at night. The carapace length, sex and cheliped configuration was recorded for each crab examined in the five Florida samples. The crusher and cutter chelipeds of most crabs in these five samples were easily distinguished by the major dentition differences described by Williams (1974). However, the crusher dentition appeared considerably eroded in a few crabs, making them superficially appear to possess double cutters. Hence, extra care was taken when examining all crabs possessing similar chelipeds (double cutters or double crushers) to insure the accuracy of their identity. Only the position of the crusher and cutter chelipeds was recorded for the hand-reared sample, and this was determined solely by location of the large blunt tooth, using a dissecting microscope. Only crabs with two functional chelipeds were considered.

RESULTS

Table II summarizes the data for all six samples. There was a significant ($X^2 = 61.8$, $P \ll 0.01$) difference among the five Florida samples for the incidence of the first two cheliped configurations in Table II. There was no significant correlation ($P > 0.3$ in each case using the X^2 test) between the first two cheliped configurations and sex in the St. Marks River, Goose Creek Bay, Trout River or Nassau Inlet samples, or when all five Florida samples were combined. While 14 of the 195 females in the Dickson Bay sample possessed a left crusher and right cutter, none of the 79 males possessed this configuration, making a X^2 comparison impossible for this sample. An analysis of variance showed significant [$F(4,1091) = 1416.3$, $P \ll 0.001$] differences in carapace length among the five Florida samples. The hand-reared sample was excluded from these statistical comparisons because of their extremely small carapace length (less than 5.0 mm), and because all individuals possessed the same cheliped configuration (crusher right, cutter left).

Although there was no clear relationship between carapace length and cheliped configuration in any of the samples when examined individually, there was a significant relationship when groups of samples were examined. Figure 1 shows the percentage (excluding crabs with double crushers and double cutters from the totals) of crabs in each size class with a right crusher and left cutter for different groups of samples. For the range of carapace lengths encountered in the three Gulf of Mexico samples (Fig. 1A) and in all six samples combined (Fig. 1C), there is a clear trend for the frequency of crabs with right crushers and left cutters to decrease as carapace length increases, and conversely for the frequency of crabs with left crushers and right cutters to increase. This trend is less clear in the Atlantic coast samples (Fig. 1B), probably due to the smaller sample sizes and narrower range of carapace lengths encountered. The fact that the hand-reared sample may

TABLE II

Cheliped configurations of 1156 Callinectes sapidus; *(L represents left; R, right).*

Sample origin	Crusher R cutter L	Crusher L cutter R	Cutter L and R	Crusher L and R	Totals
St. Marks River					
Males	148	55	12	0	215
Females	81	35	7	2	125
Total	229	90	19	2	340
Per cent	67.4	26.4	5.6	0.6	100.0
Goose Creek Bay					
Males	86	8	2	0	96
Females	71	11	3	0	85
Total	157	19	5	0	181
Per cent	86.7	10.5	2.8	0.0	100.0
Dickson Bay					
Males	71	0	6	2	79
Females	175	14	6	0	195
Total	246	14	12	2	274
Per cent	89.8	5.1	4.4	0.7	100.0
Trout River					
Males	46	9	10	0	65
Females	38	12	2	0	52
Total	84	21	12	0	117
Per cent	71.8	17.9	10.3	0.0	100.0
Nassau Inlet					
Males	35	4	14	0	53
Females	104	18	8	1	131
Total	139	22	22	1	184
Per cent	75.5	12.0	12.0	0.5	100.0
All Florida samples					
Males	386	76	44	2	508
Females	469	90	26	3	588
Total	855	166	70	5	1096
Per cent	78.0	15.2	6.4	0.4	100.0
Hand-reared					
Total	60	0	0	0	60
Per cent	100.0	0.0	0.0	0.0	100.0
All samples					
Total	915	166	70	5	1156
Per cent	79.1	14.4	6.1	0.4	100.0

have been the progeny of a single female, and probably experienced much less environmental stress than the five Florida samples, does not detract from the clarity of this relationship.

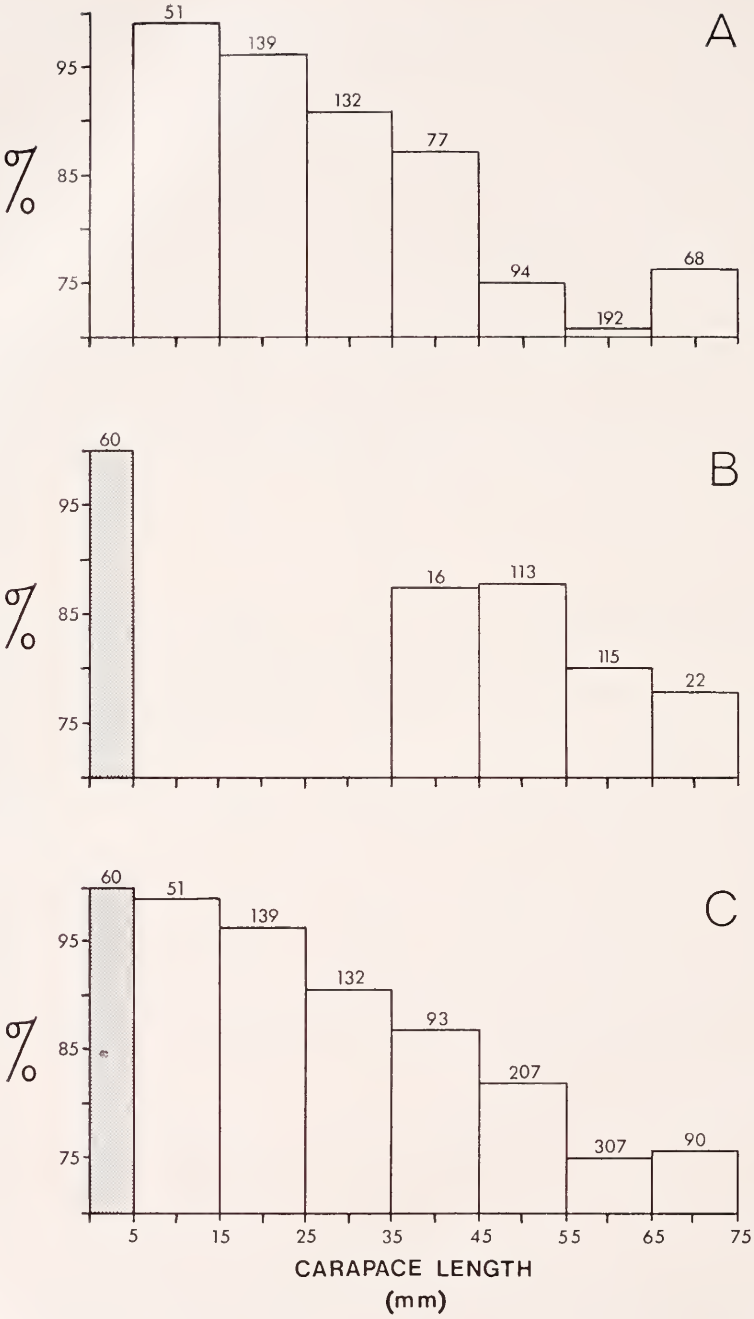
A multiple regression was run for the five Florida samples in an attempt to evaluate the effects of carapace length and sample location on cheliped laterality. However, due to the high correlation between carapace length and sample location, definite conclusions could not be drawn from this procedure.

In all 72 crabs from the five Florida samples which possessed a functional regenerated cheliped (determined by its qualitatively judged, relatively smaller size), that cheliped was a cutter. Twenty-six of these 72 crabs possessed a cutter on the opposite side from the regenerated cheliped (and hence possessed double cutters), and 46 had a crusher on the opposite side. Crabs having double cutters, one of which was regenerated, appeared significantly ($X^2 = 113.6$, $P \ll 0.01$) more often than would be expected if these two characters were not related. The fact that all 72 regenerated chelipeds were cutters suggests that double cutters actually result from the process of autotomy and regeneration. Crabs possessing double cutters, neither of which was identified as regenerated, probably commenced regeneration at an earlier date relative to their capture, and hence their chelipeds had grown more nearly identical in size. A crusher appeared on the opposite side from a regenerated cutter cheliped significantly ($X^2 = 5.56$, $P < 0.02$) more often than did a cutter.

Discussion

The data presented here suggest that some previous statements concerning cheliped laterality in *Callinectes sapidus* (and possibly in other brachyurans) have been somewhat oversimplified. The significant difference in frequency of the first two cheliped configurations among the samples could possibly be due to the different geographic locations where the samples were obtained; the significant differences in mean carapace length among the samples; or a combination of both factors. Discrete genetic differences among samples seem unlikely for several reasons. For example, the Gulf of Mexico samples were collected from closely located areas, with ample opportunity for unrestrained movement and gene exchange between adjacent areas, and for equitable settlement of pelagic larvae from parental sources. Tagging studies (Tagatz, 1968; M. Oesterling, University of Florida, personal communication) indicate that movements between the Atlantic coast sample locations, or among the Gulf coast sample locations, are well within the capabilities of adult crabs. Geographically related differences in the survival rate of crabs having different cheliped configurations could be responsible for the observed sample differences in cheliped laterality, but insufficient information is available to evaluate this possibility.

Figure 1 indicates that cheliped laterality is related to age, as indicated by carapace length, with all blue crabs apparently beginning life with a right crusher and left cutter. This trend is clear in the Gulf of Mexico samples (Fig. 1A), which almost certainly comprise a common gene pool. The observed decrease in the proportion of individuals with a right crusher and left cutter as crabs get older (larger) may be due to reversals caused by autotomy and regeneration, or merely due to spontaneous reversals at molting. Regardless of the mechanism responsible for this trend, future observations on cheliped laterality in brachyurans should in-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25
35
45
55
65
75
CARAPACE LENGTH
(mm)

clude consideration of possible ontogenetic differences in the frequency of a particular cheliped configuration.

The apparent direct relationship between the process of autotomy and regeneration and the incidence of crabs with double cutters has also been noted by Emmel (1907) in *Homarus*, and by Przibram (1931) in other brachyurans. However, the proportion of *C. sapidus* with double crushers or double cutters is much higher than in *Homarus*, where such individuals are quite rare (Herrick, 1909). The higher incidence of crabs with double cutters (and hence of autotomy and regeneration) in the Trout River and Nassau Inlet samples (Table II) is probably related to our observation that those crabs exhibited a higher frequency of cheliped damage and breakage than did crabs in the other samples.

The normal (and hence probably the most advantageous) cheliped configuration is undoubtedly one crusher and one cutter. However, in all 72 crabs possessing one functional regenerated cheliped, that cheliped was a cutter. This result certainly indicates definite selective advantage to regenerating a specific type of cheliped (a cutter) when either cheliped is autotomized. Since regenerating a cutter when a normal dimorphic crab autotomizes a cutter soon results in an almost normal crab, the selective advantage to always regenerating a cutter must accrue when a normal dimorphic crab autotomizes a crusher.

Przibram (1931) claims that a cutter is always regenerated so the crab can attain a full sized crusher and cutter again in the least amount of time. The original cutter only has to grow a little and undergo a change in dentition to become a crusher, and the regenerating cutter cheliped doesn't have to grow as much before it becomes a normal sized cutter. Since reversal of laterality is a major result in Przibram's theory, it also provides a mechanism for explaining the trend shown in Figure 1. Always regenerating a cutter regardless of which type of cheliped is autotomized could also be advantageous if spending some time with double cutters before reacquiring the dimorphism is not as disadvantageous as spending some time with double crushers. Examining the comparative versatility of crushers and cutters in feeding could provide insight into this theory. However, since a mechanism for reversal in laterality is not included in this theory, it is less parsimonious than Przibram's theory, which explains both phenomena. Neither theory accounts for the rare (0.4%) occurrence of crabs with double crushers.

Przibram (1931) also claims that complete reversal proceeds more quickly in smaller individuals of a species, and intermediate double cutter molt stages are more likely to occur in larger (older) individuals. Insufficient data is available to determine if a trend exists between the incidence of double cutters and carapace length in *C. sapidus*. However, the carapace lengths of the 70 crabs with double cutters were significantly ($z = 2.78$, $P < 0.01$) greater than those of the 1021 crabs with dimorphic chelipeds. If the incidence of double cutters is indeed

FIGURE 1. Relationship between carapace length and the percentage of crabs in each size class interval with a right crusher and left cutter for the three Gulf of Mexico samples (A), for the hand-reared sample and the two Atlantic Ocean samples (B), and for all six samples combined (C). Shaded area indicates the hand-reared sample in B and C. Crabs with double cutters or double crushers are excluded from the totals. The number of crabs in each size class interval is shown above each histogram bar.

greater in larger crabs, this may be partly due to a large cutter being able to produce enough force for most crushing tasks, making reacquisition of the dimorphism unnecessary.

The apparent reversal of laterality caused by autotomy of a crusher undoubtedly requires modifications in neural control of the chelipeds because of their different use during feeding. Regardless of whether such modifications involve actual nervous "rewiring" or merely relearning which cheliped is which, study of these changes would likely provide insight into the endogenous control of behavior in crustaceans. It would be most interesting to learn if blue crabs preferentially autotomize a cutter rather than a crusher in stressful situations. Such a response would presumably avoid a change in laterality, and would also be advantageous if a crusher is a more generally useful tool in feeding. Although crushers were found opposite regenerating cutters significantly more often than were cutters, this result could be due to either preferential autotomization of cutters, or (presuming Przibram's theory is correct) when a crusher is autotomized, to a change-over from the old cutter to a crusher before full size is attained by the regenerating cutter.

The difference in cheliped laterality between brachyuran and macruran decapods (if indeed one exists) may represent extant differences in selective pressure related to habits or habitat. Alternately, selective pressures leading to differences in laterality may only have existed in the past, and particular configurations may not provide any advantage in extant forms. Certainly the simplest explanation for the initial crusher location in brachyurans was advanced by Aristotle (quoted by Herrick, 1909), who wrote, "For it is natural to every animal to use its right side in preference to its left."

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SUMMARY

Blue crabs from five locations on the Gulf of Mexico and Atlantic Ocean coasts of Florida, USA, and juvenile crabs reared in the laboratory, were examined for the incidence of cheliped laterality (location of the crusher and cutter chelipeds). The proportion of crabs possessing the crusher cheliped on the right decreased with size (age) from 100% in the smallest crabs to about 74% in the largest crabs. Crabs with two cutter chelipeds were not uncommon and apparently resulted from regeneration of a cutter regardless of which cheliped was autotomized. Only 0.4% of all crabs possessed two crusher chelipeds.

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GAMETE-RELEASE BY 1-METHYLADENINE *IN VITRO* IN THE SEA CUCUMBER, *LEPTOSYNAPTA INHAERENS*

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It has been well established that a gonad-stimulating substance released from the nervous tissue induces shedding of gametes and oocyte maturation in starfish (Chaet and McConnaughy, 1959; Kanatani, 1964; Kanatani, Ikegami, Shirai, Oide and Tamura, 1971). The action of the gonad-stimulating substance has been shown to be indirect and is mediated by a second substance, the maturation-inducing substance, which triggers oocyte maturation and shedding of both oocytes and sperm (Schuetz and Biggers, 1967; Kanatani and Shirai, 1967). The maturation-inducing substance is produced in the follicle cells surrounding the oocytes under the influence of the gonad-stimulating substance (Hirai, Chida and Kanatani, 1973). The maturation-inducing substance has been isolated and identified as 1-methyladenine (Kanatani, Shirai, Nakanishi and Kurokawa, 1969).

In the case of the sea cucumber, *Parastichopus californicus,* Strathmann and Sato (1968) reported that the percentage of germinal vesicle breakdown in oocytes increased by treatment with an extract of starfish radial nerve. Further, Hufty and Schroeder (1974) showed that ovaries of *Parastichopus* incubated with the starfish radial nerve produce a substance which induces oocyte maturation in the starfish. The substance was chromatographically identical to 1-methyladenine (Hufty and Schroeder, 1974). On the other hand, Stevens (1970) reported that 1-methyladenine failed to induce spawning in the same sea cucumber.

Since the role of 1-methyladenine in sea cucumber reproduction remains unclear, the present study was carried out in order to determine the effect of 1-methyladenine on the induction of gamete-shedding in the sea cucumber, *Leptosynapta inhaerens.* The effects of related compounds and acetylcholine were also investigated.

A preliminary report (Koide, Ikegami and Kanatani, 1975) of this work was presented at the general meeting of the Marine Biological Laboratory, Woods Hole, Massachusetts.

MATERIALS AND METHODS

The present experiments were performed at the Marine Biological Laboratory, Woods Hole, Massachusetts, during June and July of 1974 and 1975. The sea cucumbers, *Leptosynapta inhaerens,* supplied by the Laboratory were kept in aquaria containing clean sand provided with running sea water.

The gonads were isolated in sea water and cut into pieces. The gonads generally contained motile spermatozoa and immature oocytes. However, some gonads were full of mature oocytes, and presumably spermatozoa were already shed.

The following compounds were used in order to determine their gamete-shedding activity: 1-methyladenine, 1-methyladenosine, procaine hydrochloride, adenine hydrochloride, guanine, 6-methylaminopurine, 7-methylguanine (Sigma Chemical Co., St. Louis, Missouri), 1-ethyladenine, 7-methyladenine (gifts from Dr. Nakanishi), 6-dimethylaminopurine (Cyclo Chemicals, Los Angeles), acetylcholine hydrochloride (Matheson, Coleman and Bell, Norwood, Ohio), atropine sulfate (Vitarine Co., New York) and *d*-tubocurarine hydrochloride (Abbott Laboratories, North Chicago). They were dissolved in a small amount of distilled water and diluted with sea water or dissolved directly in sea water. The pH of the test solutions was adjusted to 8.0–8.2. The sea water used was Goldstein's artificial sea water (1953).

For the assay of shedding of gametes, small fragments of the gonad which had been kept in artificial sea water were transferred to small Petri dishes containing 1 ml of test solutions, and the degree of gamete-shedding was observed for five minutes. In some cases, observation was continued for one hour to see whether germinal vesicle breakdown of immature oocytes within the gonad as well as spawning were induced.

The effects of atropine sulfate, *d*-tubocurarine hydrochloride and procaine hydrochloride on the gonadal fragments were also investigated in the absence and presence of 1-methyladenine or acetylcholine. Gonadal fragments pretreated with atropine, *d*-tubocurarine or procaine for 20 minutes were transferred to the same test solution containing 1-methyladenine (5×10^{-6} M) or acetylcholine (5×10^{-6} M).

Unless otherwise specified, at least four assays were carried out with isolated gonadal fragments obtained from different individuals at room temperature (about 23° C).

For histological study, samples were fixed in a fixative with the following composition: 5 ml of 50% glutaraldehyde, 25 ml of 8% paraformaldehyde, 20 ml of 0.2 M sodium phosphate buffer (pH 7.4) and 4 g of sucrose. They were post-fixed in 1% osmium tetroxide in 0.1 M sodium phosphate buffer (pH 7.4), dehydrated in ethanol and propylene oxide, and embedded in Epon 812. Sections were made at one μm and stained with toluidine blue.

RESULTS

Response of gonadal fragments to various purine derivatives

Among the purine derivatives tested, 1-methyladenine and 1-ethyladenine were effective in inducing shedding of sperm in isolated gonadal fragments. When the fragments were transferred to sea water containing 1-methyladenine or 1-ethyladenine at a concentration of 5×10^{-6} M, they immediately contracted, resulting in an intensive shedding of sperm (Fig. 1). Subsequently the fragments relaxed gradually and reverted to the original length. It was noted that untreated control fragments showed slow spontaneous rhythmic contractions and relaxations without any shedding of sperm. Other purine derivatives, adenine, guanine, 6-methylamino-

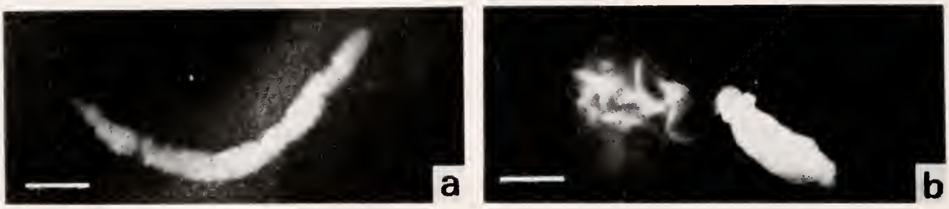

FIGURE 1. Sperm-shedding of an isolated gonadal fragment of *Leptosynapta inhaerens* treated with 1-methyladenine: *a* shows a gonadal fragment before treatment with 1-methyladenine; *b*, the same fragment, contracting and shedding sperm immediately after the treatment with 1 methyladenine at the concentration of 5×10^{-6} M. Scale bars equal 1 mm.

purine, 7-methyladenine, 7-methylguanine, 6-dimethylaminopurine and 1-methyladenosine, failed to induce sperm-shedding at the concentration of 5×10^{-6} M.

It should be pointed out that both 1-methyladenine and 1-ethyladenine were unable to induce spawning (Fig. 2) or maturation (germinal vesicle breakdown) of immature oocytes in the gonad. However, some gonadal fragments which contained mature oocytes whose germinal vesicle had already disintegrated responded immediately to 1-methyladenine (5×10^{-6} M) by shedding of these mature oocytes from the cut surface. Gonads filled with mature oocytes could be obtained from only two individuals during the period of study.

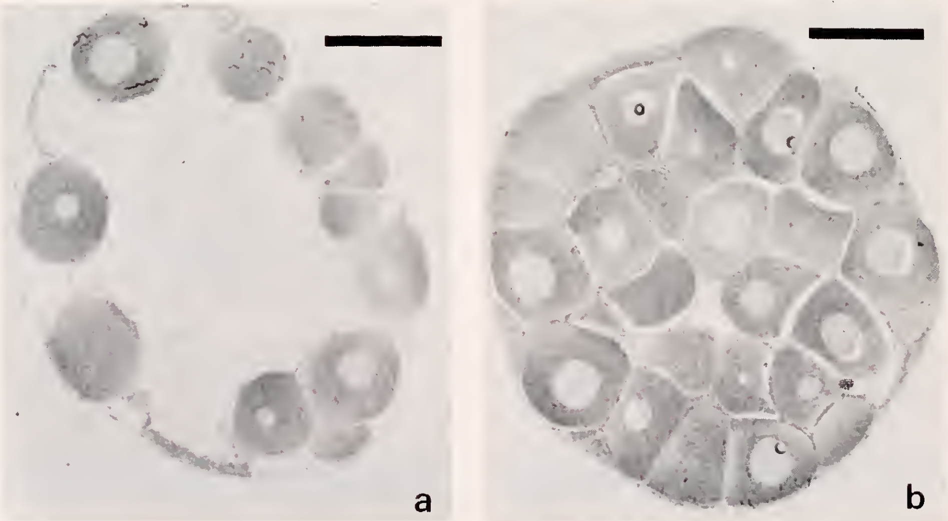

FIGURE 2. Sperm-shedding of an isolated gonadal fragment of *Leptosynapta inhaerens* treated with 1-methyladenine: *a* shows cross section of a gonadal fragment without 1-methyladenine application, showing spermatozoa in the central region and immature oocytes in the peripheral region; *b*, cross section of a gonadal fragment obtained from the same individual as *a*, which was fixed immediately after the application of 1-methyladenine (5×10^{-6} M). Scale bars equal 20 μm.

TABLE I

Effects of 1-methyladenine and 1-ethyladenine on shedding of sperm in vitro *in* Leptosynapta inhaerens *gonad.**

Experiment number†	Chemical	Concentrations					Control
		1.3 $\times 10^{-6}$ M	6.3 $\times 10^{-7}$ M	3.1 $\times 10^{-7}$ M	1.6 $\times 10^{-7}$ M	7.8 $\times 10^{-8}$ M	
1	1-Methyladenine	++	++	−	−	−	−
	1-Ethyladenine	++	±	−	−	−	−
2	1-Methyladenine	++	++	++	++	−	−
	1-Ethyladenine	++	++	++	−	−	−
3	1-Methyladenine	++	++	+	±	−	−
	1-Ethyladenine	++	+	±	−	−	−
4	1-Methyladenine	++	++	++	−	−	−
	1-Ethyladenine	++	++	±	−	−	−

* The degree of sperm-shedding was expressed as follows: ++, intensive shedding; +, intermediate shedding; ±, slight shedding; −, no shedding.

† In the same experiment number, gonads obtained from the same individual were used.

Effects of 1-methyladenine and 1-ethyladenine at various concentrations

Table I shows the effect of 1-methyladenine at various concentrations on shedding of sperm in *Leptosynapta* gonadal fragments. 1-Methyladenine was effective at concentrations above 6.3×10^{-7} M, while with various starfish species it was effective in inducing spawning of ovarian fragments and maturation of oocytes at concentrations greater than 3×10^{-7} M (Kanatani, 1969; Kanatani and Shirai, 1971). These results suggest that the minimal effective dose of 1-methyladenine required for inducing shedding of sperm in *Leptosynapta* gonad is equivalent to the dose necessary for spawning and oocyte maturation in the starfish.

1-Ethyladenine was also effective in inducing shedding of sperm in *Leptosynapta* gonads, although it was less potent than 1-methyladenine (Table I). There was a marked variation in the sensitivity of *Leptosynapta* gonads to the inducing substances when specimens of gonads taken from different individuals were used. The results were more consistent when gonads from the same individuals were used. In the present study, potencies of 1-methyladenine and 1-ethyladenine were compared on the gonadal fragments obtained from the same individual. The minimum effective concentration of 1-ethyladenine was about twice as high as that of 1-methyladenine. Again, these results are similar to those obtained with starfish ovaries with respect to the effectiveness of 1-ethyladenine and 1-methyladenine (Kanatani and Shirai, 1971).

Effects of acetylcholine

Since the time required for inducing shedding of sperm after the application of 1-methyladenine in *Leptosynapta* gonads is very short and shedding occurs within

TABLE II

Comparison of effects of 1-methyladenine and acetylcholine on shedding of sperm in vitro *in* Leptosynapta inhaerens *gonad.**

Experiment number†	Chemical‡	Concentration, M									Control
		5 $\times 10^{-6}$	2.5 $\times 10^{-6}$	1.3 $\times 10^{-6}$	6.3 $\times 10^{-7}$	3.1 $\times 10^{-7}$	1.6 $\times 10^{-7}$	7.8 $\times 10^{-8}$	3.9 $\times 10^{-8}$	2.0 $\times 10^{-8}$	
1	1-Ma	++	++	++	++	++	−	−	−	−	−
	Ach	++	++	++	±	−	−	−	−	−	−
2	1-Ma	++	++	++	++	++	++	++	+	−	−
	Ach	++	++	++	++	++	++	++	±	−	−
3	1-Ma	++	++	++	+	−	−	−	−	−	−
	Ach	++	−	−	−	−	−	−	−	−	−
4	1-Ma	++	++	++	+	−	−	−	−	−	−
	Ach	++	+	±	−	−	−	−	−	−	−

* Degree of shedding; see Table I.

† In the same experiment number, gonads obtained from the same individual were used.

‡ Abbreviations: 1-Ma, 1-Methyladenine; Ach, Acetylcholine.

five seconds, the action of 1-methyladenine resembles that of acetylcholine in shedding of gametes in sea urchins. Of the neurotransmitters studied, only acetylcholine has been convincingly demonstrated to participate in neurotransmission in sea cucumbers (Welsh, 1966). The present study was, therefore, extended to observe the effect of acetylcholine on the shedding of sperm in *Leptosynapta*. As shown in Table II, acetylcholine at concentrations above 5×10^{-6} M was found to induce instant shedding of sperm as was shown with 1-methyladenine. The minimum effective dose of acetylcholine ranged from 5×10^{-6} to 3.9×10^{-8} M depending on the material used. When compared with the action of 1-methyladenine on gonadal fragments obtained from the same individual, acetylcholine was always less effective than 1-methyladenine and 2- to 4-fold higher concentrations were required to effect shedding of sperm.

Inhibitory effect of d-*tubocurarine on 1-methyladenine and acetylcholine-induced sperm-shedding*

In order to determine the mode of action of 1-methyladenine and acetylcholine on *Leptosynapta* gonad, several drugs were examined for their ability to influence the action of 1-methyladenine or acetylcholine on gonads. It was found that atropine sulfate (10^{-5} M), procaine hydrochloride (10^{-4} M) and *d*-tubocurarine hydrochloride (10^{-4} to 6.3×10^{-6} M) did not induce shedding of sperm. After 20 minutes, the treated fragments were transferred to solutions of 1-methyladenine (5×10^{-6} M) or acetylcholine (5×10^{-6} M) containing the above drugs at the same concentrations as those used in the pretreatment solutions. The fragments pretreated with atropine or procaine shed sperm instantly as the untreated control did. *d*-Tubocurarine at greater than 5×10^{-5} M inhibited the action of 1-methyladenine or acetylcholine (Table III). The effective concentration of *d*-tubocurarine required to block shedding of sperm induced by 1-methyladenine was the same as that required to inhibit acetylcholine-induced shedding of sperm. The addition of a few drops of 0.5 M KCl to the medium containing the gonadal fragments and *d*-tubocurarine resulted in an instant shedding of sperm. These results suggest that the contractile elements of the fragments remained intact after treatment with *d*-tubocurarine.

DISCUSSION

The results of the present study show that 1-methyladenine, 1-ethyladenine and acetylcholine induced instant shedding of sperm from *Leptosynapta* gonad. However, the effects of these compounds on the sea cucumber gonads differ in some respects from those observed with starfish gonads. The shedding of oocytes or spermatozoa from fragments of starfish gonads in response to 1-methyladenine occurs about 20 to 60 minutes after the treatment. Moreover, germinal vesicle breakdown in the spawned oocytes takes place simultaneously with the spawning (Kanatani and Shirai, 1970). It is noteworthy, however, that the purine bases

TABLE III

Effect of d-*tubocurarine on 1-methyladenine- and acetylcholine-induced shedding of sperm* in vitro *in* Leptosynapta inhaerens *gonad.* The gonadal fragments were pretreated with* d-*tubocurarine at various concentrations for twenty minutes, and transferred to a solution containing* d-*tubocurarine at the same concentration as that present in the pretreatment medium and 1-methyladenine* (5×10^{-6} M) *or acetylcholine* (5×10^{-6} M).

Experiment number†	Inducing agent	Concentration of *d*-tubocurarine					Control
		10^{-4} M	5×10^{-5} M	2.5×10^{-5} M	1.3×10^{-5} M	6.3×10^{-6} M	
1	1-Methyladenine	−	−	−	−	++	++
	Acetylcholine	−	−	−	++	++	++
2	1-Methyladenine	−	−	−	±	+	++
	Acetylcholine	−	−	−	−	++	++
3	1-Methyladenine	−	−	−	++	++	++
	Acetylcholine	−	−	−	++	++	++
4	1-Methyladenine	−	−	++	++	++	++
	Acetylcholine	−	±	++	++	++	++
5	1-Methyladenine	−	−	−	++	++	++
	Acetylcholine	−	−	++	++	++	++
6	1-Methyladenine	−	−	−	++	++	++
	Acetylcholine	−	−	++	++	++	++

* Degree of shedding; see Table I.

† In the same experiment number, gonads of the same individual were used.

which induce sperm-shedding of *Leptosynapta* gonad also effect spawning and oocyte maturation in various starfish, whereas the purines inactive in inducing sperm-shedding of *Leptosynapta* were without effects on starfish gonads (Kanatani and Shirai, 1971).

Kanatani (1974) has detected the presence of 1-methyladenine in the gonads of the sea urchins, *Pseudocentrotus depressus, Anthocidaris crassispina* and *Hemicentrotus pulcherrimus* and the sand dollars, *Clypeaster japonicus* and *Peronella japonica.* Thus, evidence is gradually accumulating indicating the ubiquitous occurrence of 1-methyladenine in the gonads of Echinodermata.

Kanatani (1974) reported that 1-methyladenine treatment increased the percentage of germinal vesicle breakdown in *Anthocidaris* oocytes. In the sea urchin, *Strongylocentrotus purpuratus,* 1-methyladenine induced shedding of sperm and mature oocytes within a brief period of treatment (R. C. Cochran, University of California at Los Angeles, personal communication). As shown in this study, 1-methyladenine effectively induced shedding of sperm of *Leptosynapta* gonads but was unable to induce spawning and germinal vesicle breakdown of immature oocytes. These results suggest the possibility that a factor(s) other than 1-methyladenine may participate in the induction of oocyte maturation in sea cucumbers. However, it is also possible that the immature oocytes with which the present investigation was carried out were at an early stage of development and were incapable of responding to 1-methyladenine. It was observed with starfish that oocytes must attain a certain cell size before 1-methyladenine can be effective.

In a survey of gonads from more than one hundred *Leptosynapta inhaerens* during the breeding season, only two animals had gonads filled with mature eggs and few, if any, sperm. When these gonads were treated with 1-methyladenine, they shed their eggs instantly as was observed with sperm of other gonadal fragments. This finding indicates that 1-methyladenine may induce shedding of both mature eggs and sperm from gonads.

A failure to cause spawning of immature oocytes by the application of 1-methyladenine may be due to the presence of a thick follicular envelope which adheres oocytes together. In starfish, the disintegration of follicle cells occurs concomitantly with oocyte maturation; thus the matured oocytes are freed from adherence to each other and to the inner surface of the gonadal wall (Kanatani and Shirai, 1970). The detachment of oocytes from each other and from the gonadal wall seems to be a prerequisite for 1-methyladenine-induced spawning in the *Leptosynapta* gonad.

Acetylcholine is known to induce shedding of eggs and sperm in the sea urchin gonads (Iwata and Fukase, 1964). The occurrence and functional role of acetylcholine in sea cucumbers have been well established. Welsh (1954) demonstrated that the longitudinal body wall muscles in sea cucumbers were unusually sensitive to acetylcholine. In addition, acetylcholine-like activity was detected in the longitudinal muscles, intestine and radial nerves of the sea cucumbers, *Parastichopus californicus* and *Holothuria tubulosa* (Welsh, 1966).

Although further studies are required to determine the exact site of action of 1-methyladenine and acetylcholine before any definite conclusion can be drawn on the role of these effectors in shedding of gametes from the *Leptosynapta* gonads, the inhibition of sperm-shedding induced by 1-methyladenine or acetylcholine by *d*-tubocurarine suggests that 1-methyladenine acts on nerve cells or at the neuromuscular junction rather than directly on the muscles. Future investigations on the contraction of gonads and shedding of gametes induced with 1-methyladenine will provide new insight into the mechanism of action of 1-methyladenine in the reproductive physiology of Echinodermata.

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SUMMARY

1. The hermaphroditic sea cucumber *Leptosynapta inhaerens* does contain mature spermatozoa and immature oocytes during the breeding season. In some animals, the gonad contains eggs and few, if any, sperm.

2. Isolated fragments of ovotestes of the sea cucumber were found to shed sperm immediately upon treatment with 1-methyladenine at concentrations higher than 6.3×10^{-7} M.

3. 1-Methyladenine failed to induce both spawning and maturation of immature oocytes within the gonadal fragments, and was unable to effect germinal vesicle breakdown. Treatment of gonadal fragments containing *mature* oocytes with 1-methyladenine caused a release of these oocytes as was observed with sperm.

4. 1-Ethyladenine was also effective in inducing shedding of sperm at concentrations higher than 1.3×10^{-6} M. The following purine derivatives had no sperm-shedding effect at the concentration of 5×10^{-6} M: adenine, guanine, 6-methylaminopurine, 7-methyladenine, 7-methylguanine, 6-dimethylaminopurine and 1-methyladenosine.

5. Acetylcholine hydrochloride at concentrations greater than 5×10^{-6} M induced shedding of sperm. The sperm-shedding action of both 1-methyladenine (5×10^{-6} M) and acetylcholine (5×10^{-6} M) was completely inhibited in the presence of *d*-tubocurarine hydrochloride at concentrations higher than 5×10^{-5} M.

6. The present results suggest that 1-methyladenine acts on the gonadal nerve cells or at the neuromuscular junction of *Leptosynapta* gonads to induce contraction of the gonad, resulting in gamete-shedding.

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PRIMITIVE NERVOUS SYSTEMS. CONTROL AND RECOVERY OF FEEDING BEHAVIOR IN THE POLYCLAD FLATWORM, *NOTOPLANA ACTICOLA*

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Turbellaria are usually considered to be the most primitive animals which possess a brain. But, despite its strategic phylogenic position, very little is known about flatworm brain function. Available evidence suggests that flatworm brains have mainly a coordinatory function and that the basic motor neurones underlying specific patterns of muscular activity occur in the peripheral nervous system (Loeb, 1894; Gruber and Ewer, 1962; Koopowitz, 1970, 1975a). The African polyclad *Planocera gilchristi* is able to recognize and ingest prey in the absence of the brain, as can the Californian polyclad *Notoplana acticola*. Not all polyclads, however, possess this ability, and worms such as *Enchiridium punctatum* which have a tubular rather than plicate pharynx cannot feed unless the brain is present. One generally suspects that behavior in primitive organisms should consist of simple reflexes without much plasticity, but our analysis of feeding behavior suggests that this is, in fact, not the case. What may appear to be smooth simple behavior can be made up of a series of peripheral events with variable central control superimposed on separate systems.

This paper reports on the components of feeding behavior in *Notoplana acticola* and presents a behavioral study which precedes work on the neuronal basis of feeding in this species. As an outgrowth of this study it was found that repair to lesions in the nervous system was very rapid, and certain aspects of recovery of function have therefore also been investigated. A surprising finding is the amount of plasticity in the system. Alternative pathways can be used to convey information to the brain in the event that the normally used pathways are disrupted. A preliminary report on part of this data has already been published (Koopowitz, Silver and Rose, 1975).

Materials and Methods

Specimens of *Notoplana acticola*, approximately 1–2 cm long, were collected from tide pools at San Onofre, California. Animals were kept in 11 cm diameter finger bowls which contained 2.5 cm of sea water. Water was changed after a daily feeding of brine shrimp, *Artemia salina*. Ambient laboratory temperature was between 18° and 20° C. No aeration was required. Individuals have been kept in good condition for over six months.

All lesions were made while animals were anesthetized with a 50% solution of .36 M $MgCl_2$ in sea water. Animals recovered and would eat within one-half hour of surgery. Decerebration was performed by punching the brain out with a capillary tube (internal diameter 1 mm). It could be determined that the brain was in fact removed by inspection. In all experiments, animals were tested by holding a brine shrimp next to the part of the margin under investigation. The worms were not allowed to swallow the shrimp during testing.

Removal of part or all of the margin was used to determine the location of the chemoreceptors. Animals were then tested on the cut and noncut portions of their periphery. Observations on responsiveness were made daily until the margin had regrown.

Recovery of function experiments used a cut made through the entire body, from the midline of the animal to the margin. After lesioning, observations were made while holding a shrimp behind the cut on the lesioned side or at the same level on the control side. The response to food is a localized extension of the margin next to the shrimp and the food is gripped firmly by the margin. In an intact animal or on the control side, the animal makes an ipsilateral turn and food is transferred to the anterior margin from where it is passed back towards the mouth. An ipsilateral turn towards the food was scored as a turn (T) if the anterior margin came in contact with the food. In decerebrate animals or where food was offered behind an unhealed cut there was still localized extension and gripping of the food. No ipsilateral turn occurred and food was passed directly forward towards the mouth. Localized extension and gripping of the food was scored as a local feeding response (LFR), when it was not followed by an ipsilateral turn. In a scored LFR the worm attempted to move the food directly to the mouth, but it should be pointed out that recognition and gripping of food by the margin is essentially the same whether or not there was a turn. Results were expressed as the percentage of turns for an individual side. Usually ten readings were obtained from each side. As one side of the animal acted as control for the other side, food was offered to each side alternately. In the above experiments the food was always removed before it could be swallowed.

Sections were examined with a microscope and stained with Samuel's silver stain (1953). Samuel's stain is excellent for portraying neuronal tissue in polyclads, but unfortunately the harsh treatment administered during staining tends to affect other tissues adversely. Methods used for ultrastructural preparation and examination have been reported previously (Chien and Koopowitz, 1972).

Electrophysiological techniques involved the use of polyethylene suction electrodes. Recordings were made directly with a 3A9 verticle amplifier and a 565 Tektronix CRO. A Grass S44 electronic stimulator and Grass photoisolation unit were used to provide constant current square wave stimuli.

RESULTS

Feeding behavior in normal animals

When *Notoplana* is offered a brine shrimp at its anterior margin, that portion of the body closest to the shrimp is extended toward it. The edge of the margin grips onto the prey which is then conveyed to the midventral mouth where it is ingested. A flatworm which has been starved for one or two days will make a rapid lunge at the food and grip onto it. Animals which have been fed daily and routinely do not strike at their food. The margin closest to the food is raised above the substrate and extended towards the food. During feeding the tail end of the animal grips the substrate.

If a shrimp is offered to the posterior lateral margin of the worm, it will lift that sector of the edge and grip onto the food. This is followed by an ipsilateral turn which brings the anterior margin of the flatworm into contact with the food (Fig. 1a). The posterior lateral grip is relaxed and the food transferred to the anterior margin which grips it and then passes the food posteriorly toward the mouth. A hungry *Notoplana* will accept and eat three to five adult brine shrimp in less than five min but will not feed again for at least three-fourths to one hour. Any food offered during this satiated period will be ignored and even the initial margin extension will not occur. Toward the end of the satiated period the margin may extend and investigate the food but usually will not grip it. If it does hold

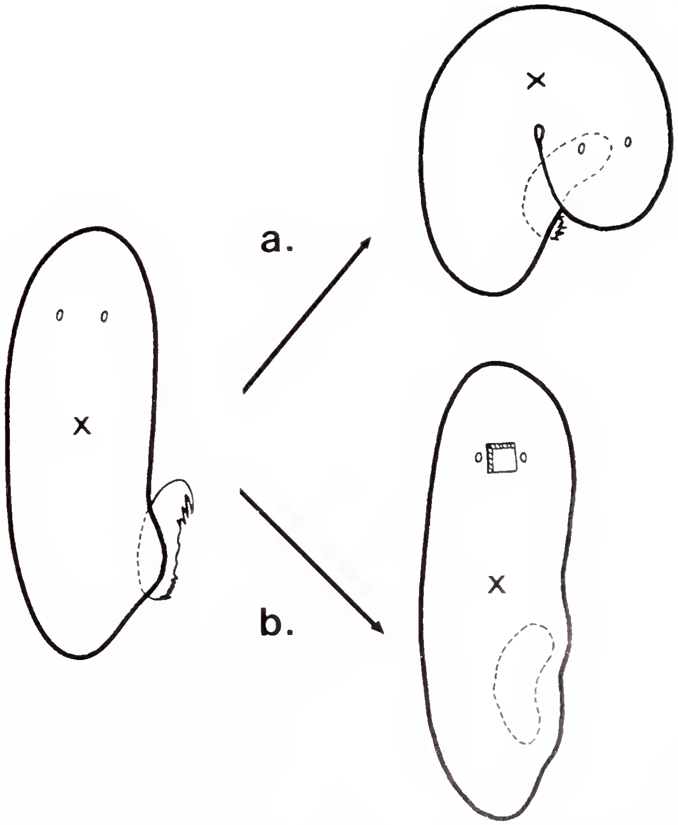

FIGURE 1. (a) Normal feeding patterns in *Notoplana acticola*. Following local extension there is an ipsilateral turn and food is transferred to the anterior part of the worm and then conveyed to the mouth. (b) Feeding in a decerebrate worm involves prolonged extension and food is conveyed directly to the mouth. Position of the ventral mouth is marked with an X.

onto the food it will not swallow it, even after the satiated period is over and after it accepts and ingests food offered to other parts of the margin.

Decerebrate feeding

There were a number of differences between normal and decerebrate animals in their feeding behavior. When shrimp was offered to the posteriolateral margin of an animal without its brain, the margin was extended and the prey held. There was no gripping of the substrate by the tail. The food held by the edge was then conveyed directly to the mouth where it was swallowed (Fig. 1b). No turning occurred. Shrimp offered to the anterior portion of the animal was accepted and passed posteriorly to the mouth. These local feeding responses (LFR) continue as long as food is offered. A decerebrate flatworm will accept so many shrimp that it cannot swallow all of them and its body will be lifted off the substrate until it is lying on top of a pile of food. Clearly, the usual satiation reflexes are not brought into play in a decerebrate animal.

A cut directly through the animal's body from the midline to the margin leaves that ipsilateral portion posterior to the cut without central control. Food offered here evokes an LFR without turning, whereas food offered to the contralateral side evokes ipsilateral turning. The portion behind the cut will continue to pass food directly to the mouth for ingestion, long after the contralateral side has become satiated. The fact that the mouth continues to swallow food provided to the cut side, after the intact portions act satiated, suggests that satiation works peripherally and blocks the LFR.

Sensory aspects of feeding

The receptors responsible for feeding behavior seem to be scattered all along the margin of the worm, as a shrimp placed in contact anywhere along the worm's margin will elicit feeding. If the entire margin is removed the animal will not feed. As a partial control to the considerable injury forced on the animal in these experiments the entire posterior third of each of a group of worms was cut off. These flatworms would feed within five minutes of being injured, whereas worms with entire margins removed did not start feeding until about five days after the surgery. Animals with only twenty per cent of the margin intact would feed if food was presented to the intact portion. Experiments with less than twenty per cent intact were not performed. It seems reasonable to assume that the receptors which initiate feeding are confined to the margin of the worm. Worms without a margin do not respond to shrimp even if the food is placed in contact with the cut edge or dorsal surface. Animals will ignore food offered to areas where only a very small portion of the margin has been cut away, as long as it does not come in contact with an intact edge. The lateral portion of the animal at the level of the eyes appears to be the most sensitive area. Feeding behavior is much more vigorous and rapid when food is presented at this region. Hungry animals will respond to vibrations in the water near them. A starved animal will attack an inert object such as a glass rod if it is moved near the animal. The worms, however, are able to differentiate between vibrations and chemical stimuli. If two glass capillary tubes, one filled with sea water and the other with shrimp homogenate, are presented to a worm together they will always choose and hold onto the tube filled with shrimp homogenate.

Normal feeding behavior requires stimulation of two sets of marginal receptors. When food is presented along the side, the lateral receptors are necessary to initiate turning. Anterior receptors are necessary for the pattern needed to convey the food to the mouth. In the following experiment a group of worms had the anterior portion of the margin excised. The next day these flatworms were presented with food to a posterior lateral margin. All the worms gripped onto the food with the posterior lateral edge and an ipsilateral turn was initiated which brought the emarginated anterior edge into contact with the food. In all cases the worms released the shrimp with the posterior margin but did not grip onto it with the anterior edge. The food was not conveyed to the mouth and these animals would not feed. They consistently moved away leaving the shrimp behind. Animals which have the nerve trunks leading to the brain cut pass food directly from the lateral margin to the mouth whereas animals which have the anterior margin removed will not feed with an LFR. This apparent inconsistency makes sense if one assumes that the central nervous system usually inhibits motor pathways involved with conveying food directly from the side to the mouth.

Previous study on the tactile system in *Notoplana* (Koopowitz (1975b) indicated that sensory information reached the brain through a diffusely conducting nerve net. The chemosensory system involved in feeding, however, appears to utilize specific routes. Cuts parallel to the margin, between 4 to 5 mm long and 1 mm from the edge of the margin, were made. The cuts were positioned in the middle of one side and were deep enough to cut right through the animal. When shrimp were offered to the margin flanked by the cut the strip of margin responded by gripping onto the food. However, no turning occurred, and the food was not conveyed to the mouth. Food offered on the uncut side evoked the turning response as would food offered on the cut side where the receptor area was not flanked by the cut.

Recovery of feeding behavior

In experiments where cuts had been made through one side of the body it was noticed that animals started to turn after a period of recovery. This was examined in more detail in the following experiment (Fig. 2a).

A series of ten worms were tested for turning towards a shrimp offered to the posterior lateral margin. All animals initially turned to both sides. In these experiments each animal was tested ten times and the form of the response was noted. The animals were then cut on one side of the body. Each lesion extended from the midline to the margin and cut right through the body. The cut was positioned halfway along the animal's length. When animals were tested for turning before the cut had healed, it was found that the control side turned most of the time and the response to food offered posterior to the cut evoked an LFR. After the cuts had healed both sides responded to food with ipsilateral turns. In order to test whether or not the nerves which were originally cut had reconnected

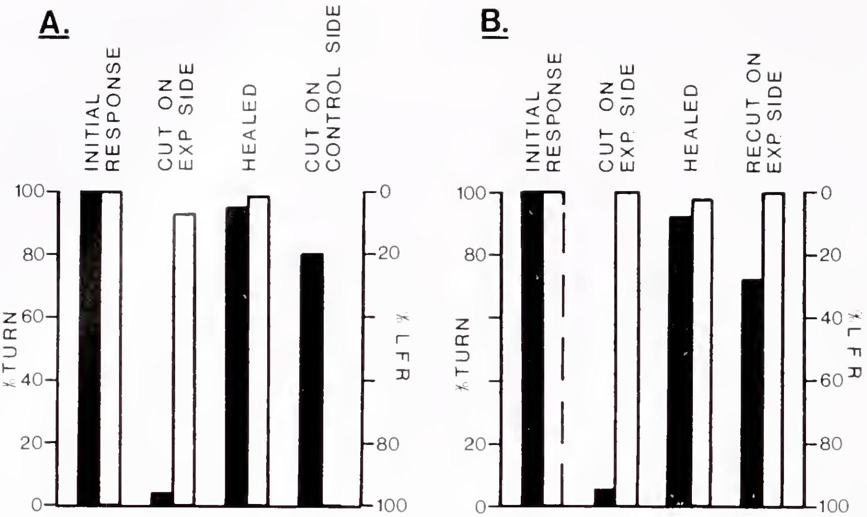

FIGURE 2. (a) Recovery of feeding behavior following lesions through one side of the animal. The black bar of the histogram is the cut side and the white bar is the control side. Treatment is written above each set of bars. Readings on healed animals were made 49 hr after the initial cut. (b) Recruitment of contralateral pathways. Experiment was the same as in (a) except that the animals were then recut on the experimental side. Healed readings were made 46 hr after the initial cut. The initial response readings were not taken on the control sides and here it is assumed that a response of approximately 100% turn would have been recorded.

in a physiologically meaningful way, another cut was then made across the control side and the animals then were retested. Now, the cut control side only responded with LFRs, but, rather surprisingly, the number of turns on the healed experimental side dropped from 94.9% to 79.8%. Close examination of the above data revealed that the drop in turning frequency was due to consistent use of LFRs by two of the worms. There appears to be three possible explanations for the reappearance of turning behavior. The nerves could have become reconnected; new or little-used tracts across the other side of the body could have been recruited; or thirdly, mechanical coupling across the cut could have stimulated some type of stretch receptor which would then have initiated turning.

Although the data in the above experiment suggested that pathways could be reestablished across a lesion, the other two possibilities were not ruled out. A further experiment was performed (Fig. 2b), similar to the first. Ten animals were tested and then cut on one side. After the cuts had healed and turning had been reestablished, the original cut was then reopened. Turning on the healed side dropped from 92.1% down to 72.1% when the cut was reopened. All the animals turned but seven worms also responded with LFRs. Whereas turning only took 1–5 sec in healed animals, it now took at least 20 sec to become initiated in reopened flatworms. Subsequent serial sectioning revealed that in all but one animal all the nerves on the healed side of the body had been resevered. The results of

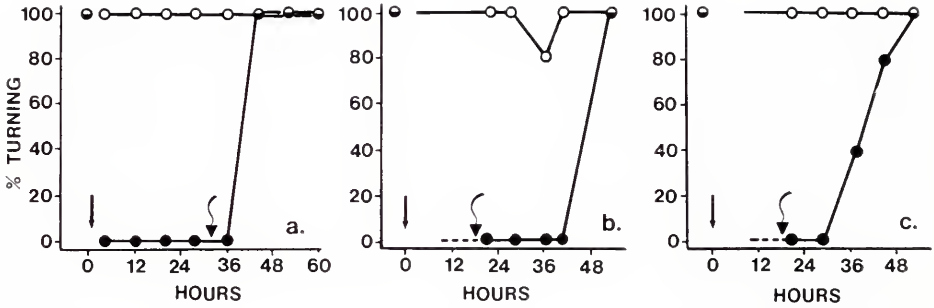

FIGURE 3. Patterns of recovery following lesions on one side. Each graph represents the performance of an individual animal. The straight arrow is the point in time when the side was cut and the curved arrow the point in time where the edges of the cut appeared to have healed. Open circles are the response of the control side and closed circles are the response from the experimental side. All axes are the percentage of turns recorded. (a) The most common pattern of healing; (b) and (c), unusual patterns seen in two individuals.

both experiments clearly show that not only can nerves rejoin to produce functional behavior but that pathways can be recruited to take over the functions of those which have been damaged. The fact that turning occurs following reopening of the cut also indicates that mechanical coupling across the cut is not a prerequisite for turning.

Time course of recovery

Recovery of ipsilateral turning behavior was followed by offering food at approximately 8 hr intervals to animals which had been cut through on one side. The most common pattern of recovery, found in six out of nine cases, is displayed in Figure 3a. Following surgery the experimental side responded with LFRs. The cut edges of the body rejoined in approximately 32 hr and the animal started to turn 100% of the time some 8 to 12 hr later. Two other patterns also occurred. In small animals the cut edges often rejoin shortly after cutting; but in one case followed, turning did not commence until 32 hr later (Fig. 3b). Usually, once turning occurs it does so nearly 100% of the time. One preparation, however, showed a gradual acquisition of turning (Fig. 3c). An important point brought out by these experiments was the fact that mechanical coupling across the cut edges seemed unimportant. There was always a delay between healing and the resumption of turning behavior.

The time course for recovery of function across the other side of the body was also followed. In this series of experiments the cut was not allowed to heal and the reappearance of turning behavior was monitored. If the edges of the cut rejoined then it was reopened as soon as noticed. These experiments turned out to be difficult to perform as worms seemed unable to tolerate all of the handling and cutting required. Of some 20 animals, 9 became completely unresponsive and could not be used. The other eleven all turned in response to food. Figure 4a is representative of the pattern of recovery seen most frequently. Animals started

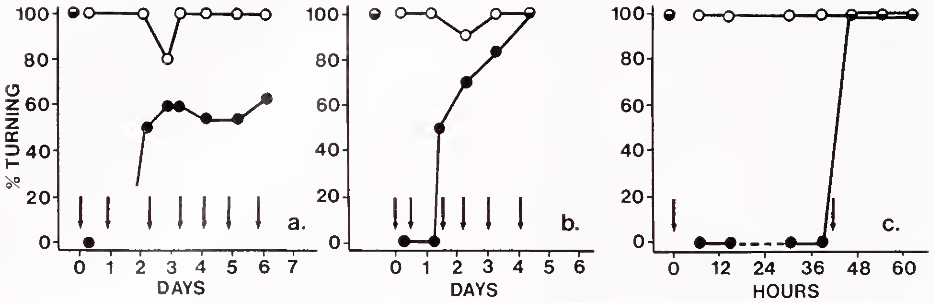

FIGURE 4. Recruitment of contralateral pathways. Each graph represents the performance of a single individual animal. In these experiments the cut was not allowed to heal. Each straight arrow represents the time when the cut was reopened. Closed circles are from the experimental side of the worm and the open circles are from the control side. (a) The most common pattern obtained. Here the animal does not achieve 100% turning during the length of the experiment. (b) A gradual improvement of performance was seen in a number of worms. (c) Abrupt acquisition of normal behavior was only seen in one preparation. The dashed portion of the line represents time during which the animal was not responsive.

turning approximately 48 hr after the original cut had been made. Characteristically these flatworms only respond with ipsilateral turns part of the time. Where acquisition of 100% turning occurs there is a gradual buildup of the effect so that it may take four or five days before this percentage is reached (Fig. 4b). In one unusual case (Fig. 4c), the animal turned 100% of the time as soon as it had recovered the behavior.

Anatomical considerations

A series of worms were examined, histologically, to see if severed nerves had rejoined. Animals were cut on one side and as soon as ipsilateral turning in response to food was reestablished the animals were fixed and sectioned. Examination of healed cuts at the light-microscope level clearly reveal nerve trunks which course across the scarred zone (Fig. 5a). Although the severed nerves realign themselves, the contents, especially of the larger trunks, tend to take on a 'bubbly' appearance (Fig. 5b). Samuel's silver stain (1953) tends to stain the outer edge of nerve axons in flatworms, and the vacuolated appearance of the trunks in the region of the scar may represent swollen tips of cut axons. Attempts to verify this or locate severed stumps at the electron-microscopy level have been unsuccessful as the scars themselves tend to be wider than the section diameters and we cannot distinguish between neurites anterior or posterior to the cut. The neuroglia in these sections are enlarged and contain much in the way of myelin bodies. Obvious degenerating axons also occur. At present one cannot recognize if two severed axons fuse together or if new processes grow out.

Conduction across healed nerves

It has been demonstrated that recovery of behavior occurs and that this is accompanied by morphological repair of the nerves. It also remains to be shown that

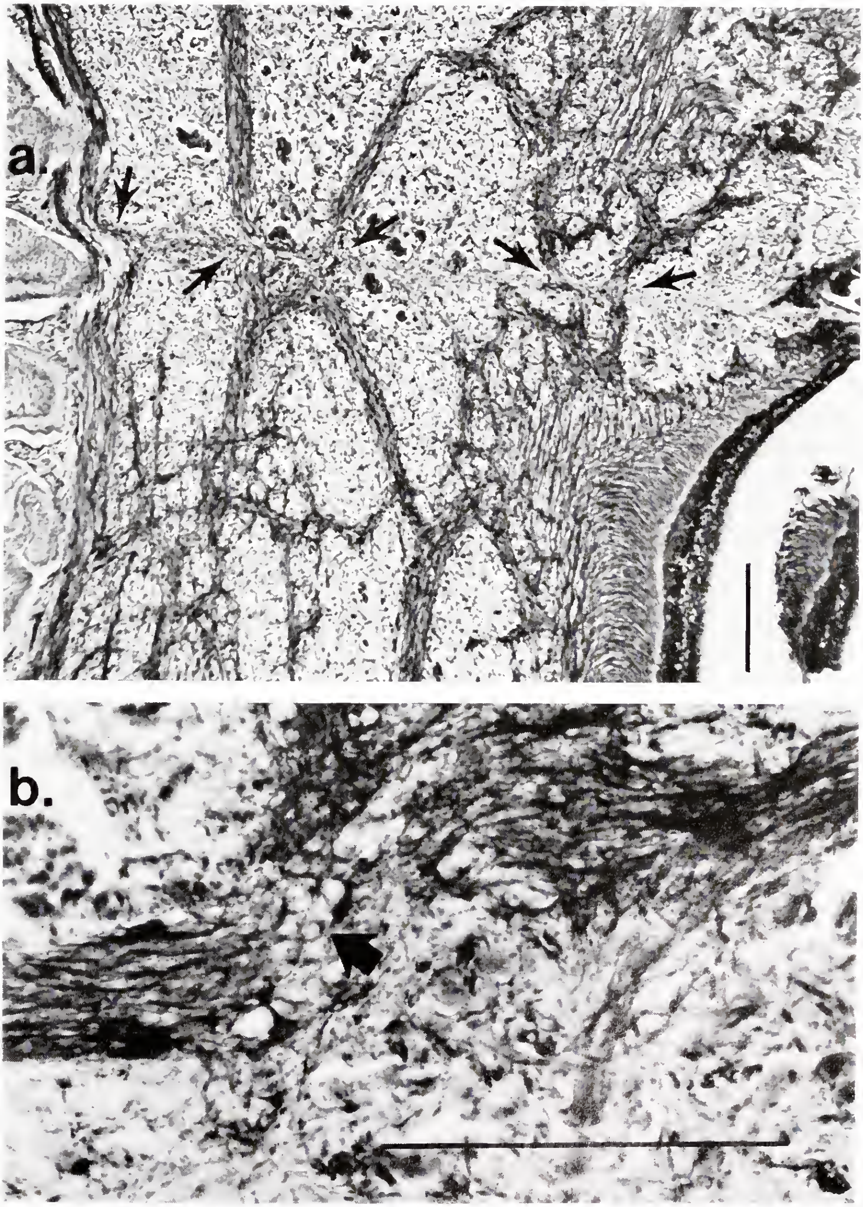

Figure 5. Section through a healed cut after turning had been reestablished. (a) Portion of the ventral submuscular plexus. Nerves in the region of the scar are indicated by arrows. (b) A single nerve trunk from another preparation to show the 'vacuolated' region of the nerve at the level of the scar, indicated by an arrow. Nerves silver-stained by Samuel's method (1953). The bar equals 200 μ.

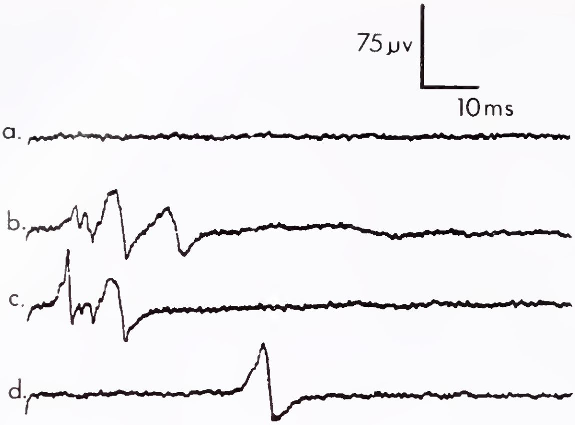

FIGURE 6. Recordings from rejoined nerve trunk. The stimulating electrode was distal to the healed part and the recording electrode proximal. (a) Single below-threshold shock of 6 volts. (b) Response to an 8-volt stimulus. (c) Response to a 10-volt shock. (d) Recording to a 10-volt stimulus after the cord had been severed between the electrodes. The potential recorded here is probably from the Mg^{++} insensitive net.

repaired nerves are able to conduct potentials across the scar. Animals which had been cut and then demonstrated recovery of behavior were selected, and the following experiment was conducted.

The flatworm was anesthetized in 50% $MgCl_2$ isotonic with sea water. Two positions on the major longitudinal nerve were exposed and cleaned so that suction electrodes could be applied to the nerve. One position for recording was close to the brain and the other for stimulating was a considerable distance behind the healed cut on a level with the genitalia. The nerve was then severed between the electrode and the brain to eliminate possible feedback from the brain. In two of three preparations, a short latency compound action potential was recorded which had a definite threshold. This potential was conducted across the healed cut (Fig. 6b and c). In all three preparations slow single unit spikes could also be elicited, but these appeared to be conducted more slowly (Fig. 6b). In the two preparations which showed compound potentials, the nerve trunk was cut between the two electrodes. Following this the compound potential disappeared but the single unit activity remained (Fig. 6d). The unit activity remained even after the cut had been continued through the contralateral cord and into the other side until a strip of margin only 1.0 mm wide remained. The activity was lost when this strip was cut too. There is no assurance or evidence that the major longitudinal trunk is used during the initiation of turning during feeding, but the experiment clearly shows that functional physiological repair of nerves can occur.

DISCUSSION

Although the neuronal pathways concerned with control of feeding behavior are not yet known, it is possible to construct a model of the kinds of systems which one might expect to find (Fig. 7). Stimulation of the receptors leads to an LFR which in a normal animal gives way to an ipsilateral turn and tail grip on the substrate. These events appear to require the brain or pathways which lead to the brain. The LFR can be inhibited or depressed in either of three ways: when the animal turns; during rapid forward locomotion; and when the animal is satiated. We have not been able to determine if these use a common pathway from the brain or if there might be separate nerve tracts involved. In a brainless animal, an LFR can be evoked at any position around the margin which suggests that numerous reflexes must be built into the peripheral plexus. Sensory pathways from the receptors appear to utilize direct pathways to the brain rather than a nerve-net such as used in conduction of tactile information to the brain (Koopowitz, 1973, 1975b). This makes physiological sense as positional information is needed to direct the anterior portion of the animal during the ipsilateral turn. Tactile information, on the other hand, appears to be a general alerting sensation, and direct positional information is in that case relatively unimportant.

The hierarchical arrangement between feeding and locomotion in *Notoplana* is of some interest. Behavioral hierarchies involved with feeding have been clearly demonstrated in the mollusk, *Pleurobranchaea* (Davis, Mpitsos and Pinneo, 1974a and b), but similar hierarchies have received little attention in the lower metazoa. What is of considerable interest here is that differential inhibition of parts of the feeding response occurs. Ditaxic locomotion, in *Notoplana*, depresses different

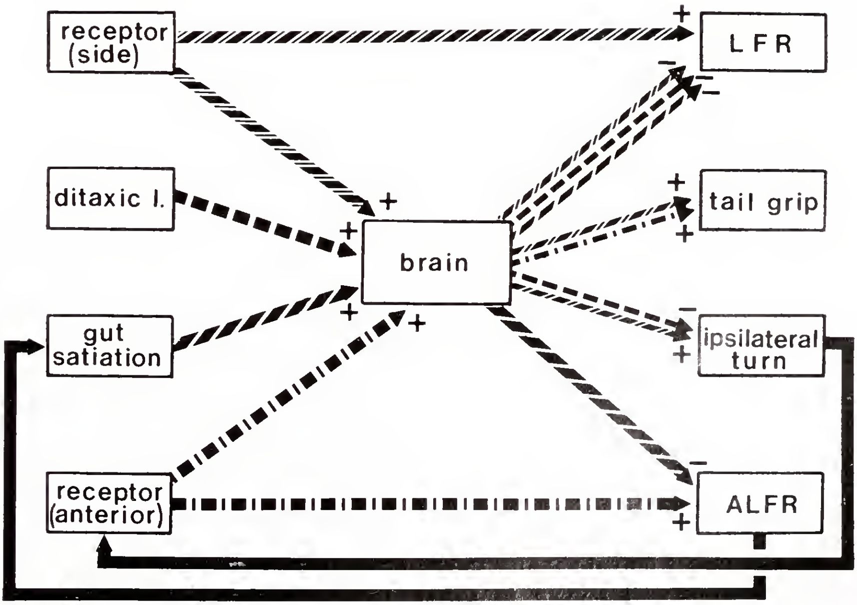

FIGURE 7. Pathways involved with feeding in *Notoplana acticola*. Pattern on the line indicates source of the pathway. Solid pathways are not neuronal but behavioral. Multiple pathways are shown rather than common pathways, as this allows one to identify the origins of the component; but there is no information on the actual anatomical pathways.

patterns of the total feeding behavior, depending on how vigorously the flatworm is moving. It is difficult to obtain quantitative data which clearly illustrate the hierarchical nature of inhibition but the following series can be observed. An animal indulging in escape locomotion to a noxious stimulus ignores all food stimuli as will other animals which merely appear to be moving rapidly for no apparent reason. On the other hand, an animal moving moderately fast will usually respond to food offered anteriorly and feed, but if food is offered posteriolaterally there is no response. A worm moving slowly will also accept food offered to the posteriolateral margin but feeds with an LFR; there is no ipsilateral turn. Non-locomoting worms nearly always turn.

Feeding behavior involves a chain of reflexes which are not necessarily neuronally connected. Quite clearly the transfer of food to the anterior part of the animal following an ipsilateral turn requires not only inhibition of the LFR but also stimulation of the anterior receptors. Similarly the initiation of inhibition of feeding with satiation is also dependent on a behavioral link in the chain rather than a neuronal connection. Animals will feed many times on small pieces of shrimp but only respond a few times when given large pieces. Little difference is seen between normal and decerebrate animal feeding when food is offered anteriorly. Consequently it is not possible to determine if the behavior following an ipsilateral turn is determined by central rather than peripheral neurones. Certainly the presence of a peripherally-based network underlying the anterior LFR does not preclude central neurones also.

Not reported in the results section are several attempts to isolate a molecule or molecules which might initiate feeding behavior, but certain comments might be useful at this point. Similar attempts to isolate a molecule that caused pharynx eversion in freshwater planarians (Ash, McClure and Hirsch, 1973) suggested that it might have a molecular weight of between 700–2000. They found that the molecule was not denatured following one min of boiling, and that it resisted both α-chymotrypsin and protease digestion. It is possible to separate out the vibration receptor response from that of the chemoreceptors by offering the animals pairs of capillary tubes, one filled with sea water and the other filled with the test chemical. The flatworms will accept seawater-filled tubes if offered alone; but if one tube is filled with brine shrimp homogenized filtrate, that will be chosen in preference to sea water. In fact, the tube with sea water will be ignored. Using ultrafiltration (Amicon UM–2 and UM–10 filters) it was determined that the molecule was larger than 1000 mol wt. We also found that boiling for 30 min had no effect. On one occasion protease digestion at 29° C destroyed most activity. Unfortunately, these results could not be repeated. The specific molecule or molecules could be further separated on a Sephadex column (G-25) and when the effluent was tested using paired capillary tubes, animals responded positively to samples in the 2000–5000 mol. wt. range. There is, of course, no assurance that the planarians and *Notoplana* might be responding to the same molecule.

Regeneration is well known in the turbellarians but many polyclads have limited powers of repair, and the brain seems to be necessary for this to take place (Olmstead, 1922). *Notoplana* will only survive for a few days if the brain is removed. Not all recovery is as rapid as turning behavior. Ditaxic locomotion in *Notoplana* appears to require the two sides of the brain intact. If the brain is bisected, animals are unable to locomote properly and recovery of locomotory behavior takes about five days to occur (J. Faisst, University of California, Irvine, personal communication). One of the major questions to be answered is whether or not the repair is due to regeneration of the axons or axonal fusion between the severed stumps.

Work on leech ganglion and cord suggest that anatomical connections formed during repair are specific between certain cells but the physiological junctions may have altered properties (Baylor and Nicholls, 1971; Jansen and Nicholls, 1972; Jansen, Muller and Nicholls, 1974). Functional repairs take considerably longer in leeches than in polyclads but axo-axonal chemical synapses appear within four days between the severed ends of axons (Fernandez and Fernandez, 1974). In crayfish Hoy (1973) has presented considerable evidence that fusion between the cut ends of axons probably occurs. Evidence for this is the rapid reappearance of behavior, within two weeks, although degeneration times are much slower. In fact severed axons in Crustacea appear to remain functional for considerable periods of time and signs of degeneration are only found in about 1% of crayfish claw nerves during the first four weeks, although the glial cell morphology associated with the severed axons had changed (Nordlander and Singer, 1972). Initial ultrastructural investigations revealed a large number of myelin bodies in the glia; this is often associated with degeneration, but without further investigations the kind of repair which occurs in *Notoplana* can not be determined.

Initially, the rapidity of recovery would seem to preclude regeneration and growth of new terminals in favor of axon fusion. If regeneration occurs at a rate of 170 μ/hr [which appears to be a maximum growth rate of axonal growth in vertebrates (Jacobsen, 1970)], then within 8 hr of the cut healing, the regenerating neurites would only have traversed 1.36 mm. As the brain usually lies about 1.5 cm anterior to the position of the cut, it would seem that growth of sensory axons to the brain could not account for repair unless growth rates were considerably faster or if synaptic connections were made close to the site of the cut. It should be pointed out that these experiments were recorded in Mg^{++} enriched saline and were close to the brain; nevertheless, potentials were transmitted to the electrode. In other neurophysiological preparations, it might be assumed that one was recording from continuous axons because synapses should be blocked by the Mg^{++} ions. The nerve net in some polyclads, such as *Freemania* and *Notoplana* (Koopowitz, 1975b), is insensitive to magnesium ions although certain central and neuromuscular junctions are blocked. It is possible, therefore, that Mg^{++} insensitive connections were formed. An interesting observation obtained while recording from the ventral longitudinal nerve trunk was the presence of the diffusely conducted spike. Although this potential cannot be tied to a particular behavior it shows that potentials can be fed into the longitudinal tract through the diffusely conducting system, and that these function in high Mg^{++} concentrations. Whether this type of system is involved with rerouting of sensory information across to the other side of the body is unknown. The possible presence of Mg^{++} insensitive synapses precludes simple interpretation of the data, and deciding if recovery involves axon fusion or regeneration or both must await further work.

SUMMARY

1. Feeding behavior in *Notoplana acticola* involves a series of local responses which are under central control. Behavior involves recognizing food, gripping with the tail and turning towards the prey. Food is then conveyed to the mouth and swallowed.

2. Worms are still able to ingest food in the absence of the brain using local reflexes.

3. Central control is abolished in areas posterior to cuts through the body wall.

4. Functional recovery of behavior occurs in a minimum of 8 hr following closure of the wound.

5. Anatomical fusion of cut nerves occurs and conduction of potentials can be demonstrated across the healed cut regions of major nerves.

6. Recovery of function also occurs in approximately 36 to 48 hr if the cut is not allowed to heal suggesting that new or normally unused pathways can be recruited.

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THE BLOOD OF *ASCIDIA NIGRA*: BLOOD CELL FREQUENCY DISTRIBUTION, MORPHOLOGY, AND THE DISTRIBUTION AND VALENCE OF VANADIUM IN LIVING BLOOD CELLS

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The vanadium concentrating ability of some tunicate species and their blood cells, particularly those cells which contain the vanadium, have long been the subject of investigation (Senozan, 1974). Theories of the function of this metal have been presented, including its possible roles in oxygen binding (Carlisle, 1968), tunicin synthesis (Endean, 1960) and antimicrobial defenses (Brown and Davies, 1971); but as yet there is no widespread agreement supporting one of these suppositions (Goodbody, 1974).

To establish the function of vanadium in the blood of ascidians, the morphological and physiological aspects of the blood should be well characterized. However, although many investigators have studied the nature of the metallic, organic and protein contents of the blood cells, procedures were employed which resulted in extensive cell lysis (Webb, 1939; Califano and Caselli, 1949; Boeri and Ehrenberg, 1954; Bielig, Bayer, Dell, Rohms, Mollinger and Rudiger, 1966), and therefore introduced significant denaturation and artifact effects. Consequently, this study was undertaken to determine blood cell frequency distribution, morphology, and the distribution and valence of vanadium in living *Ascidia nigra* blood cells. The study uses physical and chemical techniques which do not require lysis of the blood cells, *e.g.*, nuclear magnetic resonance (Carlson, 1975). Degradative techniques are used only to obtain reliable quantitative data on the total vanadium content of the blood. This study will therefore aid in understanding the function of vanadium in the blood.

MATERIALS AND METHODS

Ascidia nigra specimens were collected on the coastline and surrounding reefs of Bermuda by the Bermuda Government Aquarium. They were placed in plastic bags filled with tropical sea water, packed in insulated styrofoam boxes and shipped air freight to Boston. Since the specimens were situated in unaerated water for at least eight hours, they were immediately transferred to two continuously aerated, heated 50-gallon aquaria with freshly filtered sea water and a biological filtration system upon arrival at the New England Aquarium. Water quality was monitored by routine tests and maintained with frequent water changes at the following specifications: temperature, 24.5 ± 2.5° C; salinity (hand refractometer, American Optical Corporation), 3.25 ± 0.05% (~ 0.55 M NaCl); pH (meters, models 175 and 205, Instrumentation Laboratories, Inc., Boston), 7.9 ± 0.3; dissolved oxygen (Winkler method), 6.7 ± 0.8 ppm; ammonia (Gilbert and Clay, 1973; NH_3 electrode model 95-10, meter model 407, Orion Research, Inc., Cambridge), 154 ± 84 ppb (large range due to the varying number of specimens in the tanks during a particular measurement); nitrite (Strickland and Parsons, 1955), undetected.

Cultures of *Skeletonema costatum* and *Dunaliella euchlora* grown at room temperature under fluorescent light in supplemented (half-strength) sea water, type-f (Guillard and Ryther, 1962), were added to the aquaria for food on alternate days. Unhealthy specimens, evidenced by a discoloration of the tunic, were removed and discarded as they appeared. Usually, 10–20% of the tunicates had to be removed within one week after their arrival from Bermuda, but the remainder of the specimens remained healthy until sacrificed, up to two months later.

The dissection procedure for blood withdrawal was as follows. The specimen was removed from the aquarium and gently squeezed to remove the sea water from the body. Sections of the tunic external to the heart tube were removed in thin slices until the heart was exposed. During this slicing process, spots of blood would appear on the sectioned surface of the tunic, but blood loss was insignificant, and this method permitted a quick yet careful dissection to be carried out. The heart tube was cut, and blood that welled up in the cut-away portion of the animal was collected with a plastic syringe (sterile Stylex syringe, Pharmaseal Laboratories, Glendale, California, fitted with Lancer Precision Pipette tips, Sherwood Medical Industries, St. Louis, Missouri). The openings of the plastic pipette tips were enlarged to prevent damage to the blood cells during the blood withdrawal process. If cell rupture does occur, probably resulting in the release of sulfuric acid, which is found in significant concentrations in the blood cells of other ascidians (Webb, 1939; Endean, 1955, 1960), the pH is observed to descend as low as 2–3, in agreement with past observations (Bielig, *et al.*, 1966; Senozan, 1974) and the blood cells become dark green-brown in color. Only blood samples which retained a bright yellow-green color were used for experimentation.

Plasma was separated from blood cells by centrifugation (Centrifuge model HN-S, International Equipment Co., Needham Heights, Massachusetts) for pH, salinity, and spectrophotometric analysis (Gilford Spectrophotometer Model 240, Gilford Instrument Laboratories, Inc., Oberlin, Ohio).

Using standard techniques (Bauer, 1970; Curby and Winick, 1974), whole blood was analyzed for total cell count (MK-40 hemocytometer, General Diagnostic, Bridgeport, Connecticut), cellular volume (Clay Adams microhematocrit tubes and putty, International Centrifuge Model MB), hemoglobin (iron) content (cyanomethhemoglobin, MK-40 system), and cell size (Olympus scales and microscope, Japan; Curby Bio-Detector, Grumman Corp.). Cell morphology was examined by study of blood smears (Olympus microscope) and photomicrographs (Zeiss Model RA microscope; Nikon M-35S camera) of blood slides.

Spectrophotometric analysis of whole blood was conducted in two experiments. In the first, blood was withdrawn, suspended in glycerol (Glycerol, spectro, lot # 691-A, Eastman Kodak, Rochester, New York), and analyzed in the Gilford instrument in the presence of air. Both fresh and 4-day old blood was analyzed in this manner. In the second method, blood was withdrawn in a glove-box in a nitrogen (Suburban Welders Supply Co., Inc., Ashland, Massachusetts) environment, suspended in deaerated glycerine (Glycerine, lot #713671, Fisher Scientific Co., Fairlawn, New Jersey), and analyzed spectrophotometrically (Beckman Model 25, Fullerton, California) under argon gas (Suburban Welders Supply Co., Inc.).

Electron paramagnetic resonance studies using a Strand Labs Model 602B X-band esr spectrometer (Tuttle, Danner and Graceffa, 1972) were carried out in two experiments. The first was on blood withdrawn in a glove-box (New England Aquarium) in a nitrogen (Medical-Technical Gases, Inc., Medford, Massachusetts) environment and collected in a 50 microliter micropipet (Clay Adams, Parsippany, New Jersey) sealed with plastic clay (Seal-Ease, Clay Adams). In the second experiment, esr analysis was done on blood withdrawn in the presence of air, which had been refrigerated (6° C) for at least two weeks in a rubber-stoppered glass tube (Vacutainer, Becton, Dickinson and Co., Fairlawn, New Jersey).

Nuclear magnetic resonance studies using a modified Brucker model WH-90 nmr (Redfield and Gupta, 1971) were carried out on blood cells placed in a 5 mm glass tube (507-PP, Wilmad Glass, Buena, New Jersey). This tube was inserted into a 10 mm glass tube (513-IPP, Wilmad Glass) containing a DSS (sodium 2,2-dimethyl-2-silapentane-5-sulfonate) reference (Wilmad Glass, New Jersey).

Density separation of the blood cells was accomplished by centrifuging (400 *g*, 15 minutes; International Equipment Co., Centrifuge Model 80120-B) blood diluted with 2.8% saline (Abbott Laboratories, Needham, Massachusetts) which had been layered above 1.5 mol of a Lymphoprep (9.5% Sodium Metrizoate, 5.6% Ficoll; Nyegaard and Co., Oslo, Norway) medium. This is a standard medium used for the separation of human leukocytes from other type blood cells (Boyum, 1968).

Histological analysis for vanadium was accomplished through the use of osmium tetroxide (Stevens Metallurgical Corp., New York; reagent, 1800 ppm, 0.18 g OsO_4 dissolved in 100 ml 2.8% NaCl and dilute NHO_3) applied as a stain to microscope slides of whole blood. Quantitative determination of vanadium in saline solutions of cell lysates was accomplished with a standard technique (Ladd, 1974) of atomic absorption spectroscopy (Instrument Laboratories Model 153, dual beam atomic absorption spectrophotometer).

RESULTS

Plasma characteristics

Plasma pH is essentially neutral (6.7) in *Ascidia nigra*, as it is in the plasma of *A. aspersa*, *A. mentula*, *A. fumigata*, and *A. mammillata* (Webb, 1939). Plasma salinity (3.30%) approximates that of the aquaria sea water in which the specimens resided. The ultraviolet-visible (uv-vis) absorption spectrum of a plasma sample (pH 3.1, due to rupture of some blood cells) which was yellow-green and colloidal in appearance, diluted 5:1 with distilled water (resultant pH 3.7) blanked against

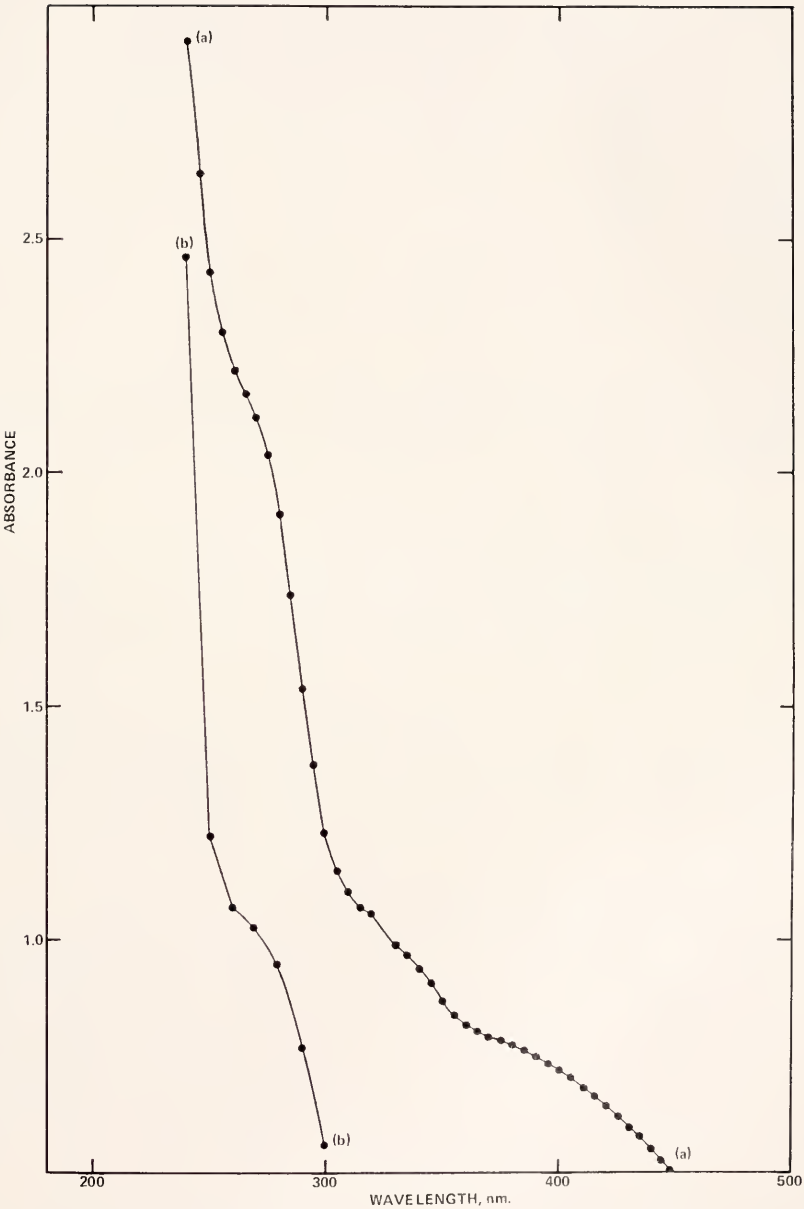

FIGURE 1. Absorption spectra of (a) plasma and (b) 1.0 N H_2SO_4, against a distilled water reference.

TABLE I

Differential cell counts of whole blood. Specimens 1 and 3 were the youngest of the five. Differential counts of the blood cells and volumes in the Lymphoprep layers (Tables III, IV, VI) were not obtained; therefore, Specimen 5 does not appear in those Tables.

Cell type	Large smooth	Small smooth	Green globular	Orange	Blue
Specimen 1	26%	39%	29.5%	6%	0%
Specimen 2	20.5	62	13.5	2	2
Specimen 3	40	33	14	12	0
Specimen 4	33.7	33	33	0.3	0
Specimen 5	51	38	9.5	1.5	0
Average	34.2	41.0	19.9	4.4	0.4
Absolute s.d.	11.9	12.1	10.6	4.8	0.9

distilled water, is without distinct peaks, except for a shoulder in the 260–280 nm range (Figure 1a) which is due, perhaps, to absorption by the released sulfuric acid (Figure 1b).

Blood cell characteristics, morphology, and frequency distribution

Both total blood cell count and per cent cellular volume are found to be in agreement with previously reported results for *A. nigra* (Vallee, 1967): 53,800 cells per cu mm and 1–2% cells, respectively. There is a constituent of the blood which, by the cyanmethemoglobin method, corresponds to 5.0–5.5 gram % hemoglobin; or to an iron concentration, if all the iron is in this form, of 80 μM. Cell sizes range from 2–10 microns, slightly larger than human erythrocytes, but smaller than granulocytes (Bauer, 1970). Various cell types that are observed on unstained slides correspond to earlier morphological descriptions (George, 1930, 1939; Endean, 1955, 1960), but differential counts (Vallee, 1967) of these cell types seem subject to considerable variation, due, perhaps, to the ages of the individual specimens examined, like the nonpathological differences seen in the blood of humans of different ages (Bauer, 1970).

There are four main groups of blood cells: large smooth, small smooth, green globular and orange. Large smooth cells are primarily single nucleated cells, 6–8 microns (George's vesicular signet-ring cells) and fewer "ghost" cells, 8–10 microns. Small smooth cells are primarily colorless morula types with 2–5 lobes, fewer small ghost cells, 3–4 microns, and still fewer small ghost cells with a single appendage (probably amoebocytes). Green globular cells, corresponding to George's green cells or vanadocytes are present in various sizes and with varying numbers of lobes, suggestive of different cellular developmental stages. Orange cells are those with a very bright orange pigment.

Cells that are infrequently found are blue cells, brown cells, and cells with basophilic-like stippling, 8–10 microns in diameter. As to the relative frequency of occurrence of the four main groups of cells (Table I), large smooth, small smooth, and green globular cells are significantly more abundant (34.3%, 41.0%, and 19.9%, respectively) than the orange cells, which are the least (4.4%) abundant. Younger specimens seem to have higher percentages of orange cells (6% and 12%), which is significantly higher than figures previously reported (Vallee, 1967).

The blood of a young specimen was observed to develop two layers of cells on standing in a glass tube for five to ten minutes: an orange top layer consisting of large smooth, small, and orange cells, and a yellow-green "pellet" primarily composed of green globular cells (Table II). Total cell counts for each of these two layers were tabulated on the Curby Bio-Dectector: the top layer having 14,508 cells per cu mm; the pellet, 64,341 cells per cu mm. If blood samples, therefore, are not adequately agitated to produce homogeneous mixtures, this settling phenomenon (which is observable in human blood only over a period of several hours) may account for two findings: the variation in differential cell counts (Table I) of whole blood, and the wide range of total cell counts reported for *A. nigra* [31,600–79,390 cells per cu mm (Vallee, 1967)] and for *Pyura stolonifera* [18,250–68,200 cells per cu mm (Endean, 1955)]. Another possible source of this variation is the possibility that it stems from variations in the rate of influx to the blood of undifferentiated or partially differentiated blood cells and/or variations in the rate of efflux from the blood of fully differentiated or senescent cells.

Although the results of histological staining will provide more conclusive evidence, the presence of various transitory stages of cells in unstained blood (as well as the results of the Lymphoprep density centrifugation, discussed below) suggest a maturational sequence which would be in fair agreement with past hypotheses (Endean, 1960; Kalk, 1963): the large smooth cell becomes a colorless morula-shaped cell, which changes into the green vanadocyte.

Ultraviolet-visible absorption of blood

The uv-vis absorption spectrum of intact blood cells suspended in glycerol (glycerine), blanked against the same, demonstrates the effect of exposure to air,

TABLE II

Differential cell counts of blood allowed to stand five to ten minutes.

Cell type	Large smooth	Small smooth	Green globular	Orange	Blue
Top layer	26%	56.5%	5.5%	12.5%	0%
"Pellet"	26	14	52	5.5	2
Number of cells per cu mm, and as % of 53,800					
Top layer	13,988	30,397	2,959	6,725	0
"Pellet"	13,988	7,532	27,976	2,959	1,076
Weighted average	13,988	18,965	15,468	4,842	538
Per cent	26.0	35.3	28.8	9.0	1.0
Per cent ranges (Table I)	22.3–46.1	28.9–53.1	9.3–30.5	0–9.2	0–1.3

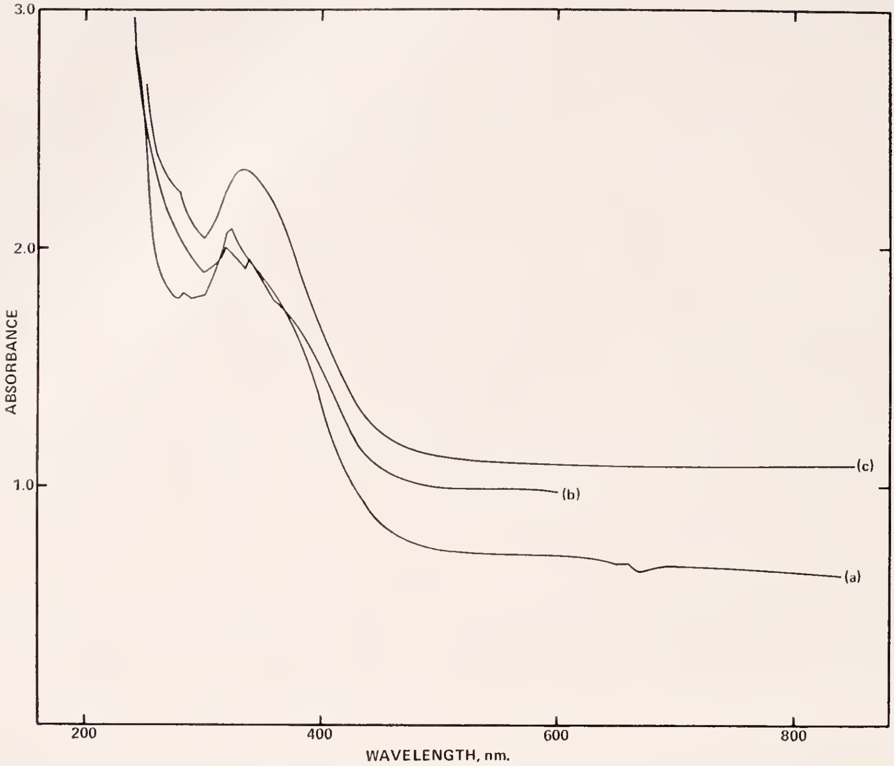

FIGURE 2. Absorption spectra of blood cells. Curves (a) and (c) were taken immediately after dissection; (a) for aerated and (c) for unaerated blood. Spectrum (b) is for four-day old refrigerated blood. In each case the cells were suspended in glycerol and blanked against the same.

as well as the effect of cell lysis. Blood which is isolated from atmospheric oxygen (Fig. 2c) shows an absorbance maximum at 335 nm and a shoulder absorbance in the vicinity of 280 nm. Blood which is allowed to come into contact with air (Fig. 2a) shows a shift of its absorption maximum to 325 nm. Four day old blood, exposed to air and refrigerated (Fig. 2b) has two absorbance maxima at 325 and 340 nm. Additionally, the positions of all of the above intact blood cell absorption maxima differ significantly from maxima reported for different forms of lysed blood cell solutions at 275, 280, 300, 420, and 610 nm (Bielig, Bayer, Califano and Wirth, 1954).

Magnetic resonance analysis of blood

Electron paramagnetic resonance (epr). The eight-line spectrum (Carrington and McLachlan, 1967) of VO^{2+} ion in acidic medium was clearly visible in a 0.001 M vanadyl sulfate solution at 20° C. The epr line spectrum of V^{3+} has not been observed in solution as it is too broad (Carrington and McLachlan, 1967).

Blood extracted in a nitrogen environment shows no epr signal. Air-oxidized, two week old blood yielded a VO^{2+} signal roughly comparable to that of the millimolar reference. Vanadium present in the blood cells is therefore in the +3 valence state. The relatively low concentration of vanadium(IV) in the aged sample is probably due to the fact that oxidation proceeds to the +5 state. The presence of V(III) in *A. nigra* is in agreement with the conclusion (Swinehart *et al.,* 1974) that in vanadium-concentrating species (order: Enterogona) the +3 valence state is more common among species of sub-order Phlebobranchiata, of which the Ascidiidae are members (Millar, 1966).

Nuclear magnetic resonance (*nmr*). The proton nmr spectrum of a centrifuged blood sample was taken at 22° C. The spectrum consists of two resonance lines: a very strong, essentially Lorentzian signal approximately 5 ppm downfield from a DDS (sodium 2,2-dimethyl-2-silapentane-5-sulfonate) reference; and a weaker, apparently Gaussian signal with a peak about 20 ppm from the DDS reference line. These features are consistent with an earlier report on the ^1H nmr spectrum of living blood cells of *A. ceratodes* collected on the Pacific coast (Carlson, 1975). The previous assignment of the strong signal to plasma water and the weaker, 20 ppm resonance to vanadophore solvent water broadened by paramagnetic V(III) is also supported by the *A. nigra* spectra. However, the width of the line we have measured appears to be considerably narrower; the full width at half height is approximately 600 Hz. If the nuclear relaxation process is controlled by the rate of chemical exchange, as it is for dilute aqueous V^{3+} (Chmelnick and Fiat, 1972), the line should narrow with decreasing temperature, and the *A. nigra* nmr spectrum at 22° C. would then be consistent with that of *A. ceratodes* at 33° C.

Taken together, the epr and nmr studies indicate the existence of a tripositive, partially complexed vanadium species in the blood cells. The inference of further conclusions on the structure of the vanadium complex from the resonance data does not seem to be justified at this time.

Lymphoprep density centrifugation of blood

Density centrifugation of saline-diluted blood on Lymphoprep medium results in the formation of five layerings (Fig. 3a) of fairly uniform volumes (Table III)

Table III

Results of lymphoprep density centrifugation (volumes in ml).

Specimen	Volume whole blood	Volume saline added	Total volume	Cell band volumes and percentages of total volume									
				1	%	2	%	3	%	4	%	5	%
1	0.70	4.30	6.5	4.50	69.2	0.25	3.8	0.60	9.2	0.80	12.3	0.10	1.5
3	0.50	3.00	5.0	1.83	36.6	0.67	13.4	0.67	13.4	0.11	2.2	0.07	1.4
5	0.30	1.80	3.6	2.48	68.6	0.73	20.3	0.51	14.2	0.22	6.1	0.05	1.4
Average					58.2		12.5		12.3		6.9		1.4
Absolute s.d.					18.7		8.3		2.7		5.1		0.1
2	1.30	3.70	6.5	4.70	72.3	0.35	5.4	1.15	17.7	0.13	2.0	—	—

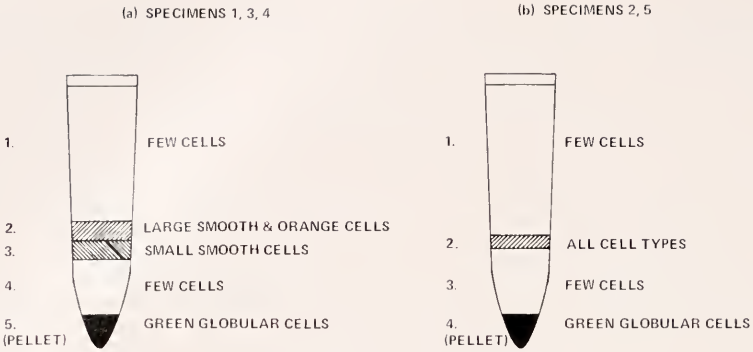

FIGURE 3. Results of lymphoprep density centrifugation.

or four layerings (Fig. 3b). Layers 2 and 3 (which consist of lymphocytes when human blood is centrifuged in Lymphoprep) are composed of large smooth and orange cells, and small smooth cells, respectively. Layer 5, the pellet (which would consist of erythrocytes of human blood), is primarily composed of green globular cells (Table IV). Note that in this and succeeding tables layer 2 of specimen 4 will be considered with layers 2 and 3 of specimens 1, 3, and 4, since the former did not differentiate the lower density cell layer. The relative frequencies of the blood cells in a particular layer and the layer preferences of the cells (Table IV), indicative of the density differences, correspond to the suggested maturational sequence of large smooth to small smooth to green globular.

Vanadium content of blood

Application of the OsO_4 reagent (or a 50% dilution of the reagent with 2.8% saline) produces blackening of the interiors of the green globular cells, with no observed discoloration of any of the other cell types or plasma in slides of whole blood. It seems probable, therefore, that trivalent vanadium in these green globular cells (green cells, vanadocytes) is the cause of the chemical reduction of OsO_4 (George, 1939; Webb, 1939).

Overall vanadium concentrations of whole blood samples determined by atomic absorption spectroscopy (Table V) range from 2.54–23.44 mM, with an average of 10.73 mM. Vanadium concentration of the Lymphoprep density centrifugation layers (Table VI) corresponds directly to the green globular vanadocyte layer preference (Table IV) of Layer 5 > Layer 2 > Layer 3; and some of these cells could be reasonably expected to be found in layers 4 and 1.

DISCUSSION

Within a short time after cell rupture, the lysate has a different hue from the intact cells. The specific color obtained is a function of the age and degree of oxidation of the solution. For example, the yellow-brown lysate solution previously reported (Boeri and Ehrenberg, 1954) is probably a hydrolytic dimer of

TABLE IV

Differential cell counts of lymphoprep density centrifugation layers. Note that the relative frequencies of the cell types in the layers are: layer 2, large smooth > orange > green globular > small smooth; layer 3, small smooth > green globular > orange > large smooth; layer 5, green globular > large smooth > small smooth > orange. The layer preferences of the cells are: large smooth, 2 > 5 > 3; small smooth, 3 > 5 > 2; green globular, 5 > 2 > 3; orange, 2 > 3 > 5.

Cell type	Large smooth	Small smooth	Green globular	Orange
Specimen 1, layer 2	47%	9.5%	4%	40.5%
Specimen 2, layer 2	14	15	37.5	33.5
Specimen 3, layer 2	16	6	33.5	44.5
Specimen 4, layer 2	71.5	22.5	5	1
Average	37.1	13.3	20.0	29.9
Absolute s.d.	27.4	7.2	18.0	19.8
Specimen 1, layer 3	7.5	63.5	5	24
Specimen 2, layer 2	14	15	37.5	33.5
Specimen 3, layer 3	13.5	57	23.5	6
Specimen 4, layer 3	7	88.5	3.5	1
Average	10.5	56.0	17.4	16.1
Absolute s.d.	3.8	30.5	16.2	15.2
Specimen 1, layer 5	22.5	8	68	1.5
Specimen 2, layer 4	20.5	32	46	2
Specimen 3, layer 5	43.5	5	47.5	4
Specimen 4, layer 5	25.5	22.5	50.5	1.5
Average	28.0	16.9	53.0	2.3
Absolute s.d.	10.5	12.7	10.2	1.2

vanadium(III) (Newton and Baker, 1964). This and other examples of dissociation between metal and complexing ligand, oxidative reactions of metal and ligand, and degradation of protein show that the correct nature of the vanadium

TABLE V

Vanadium content of whole blood.

Specimen	[V], millimolar	ppm, or milligrams/liter
1	23.44	1195
2	11.82	603
3	12.15	620
4	3.72	190
5	2.54	130
Average	10.73	548
Absolute s.d.	8.38	427

TABLE VI

Vanadium content of Lymphoprep density centrifugation layers. Per cent cells assumes a cell volume of 320μ³ (Webb, 1939). Average (−2) is the average excluding Specimen 2 data.

Specimen	Layer [V], mM	Layer volume ml	Per cent total volume	Vanadium concentration micromoles	Per cent total V	Layer cell count per cu mm	Per cent cells
Specimen 1, layer 1	0.0	4.50	69.2	0.0	0.0	654	0.021
Specimen 2, layer 1	0.0	4.70	72.3	0.0	0.0	242	0.008
Specimen 3, layer 1	0.54	1.83	36.6	0.99	13.81	175	0.006
Specimen 4, layer 1	0.41	2.48	68.8	1.02	40.32	109	0.003
Average (−2)	0.24	3.38	61.7	0.50	13.53	295	0.010
Absolute s.d.	0.28	1.44	16.8	0.58	19.01	245	0.008
Average	0.32	2.94	58.2	0.67	18.04	313	0.010
Absolute s.d.	0.28	1.39	18.7	0.58	20.49	297	0.009
Specimen 1, layer 2	12.83	0.25	3.8	3.21	23.67	12278	0.39
Specimen 2, layer 2	7.62	0.35	5.4	2.67	13.50	6646	0.21
Specimen 3, layer 2	2.72	0.67	13.4	1.82	25.38	2930	0.09
Specimen 4, layer 2	1.19	0.73	20.3	0.87	34.39	2816	0.90
Average (−2)	6.09	0.50	10.7	2.14	24.24	6168	0.20
Absolute s.d.	5.26	0.24	7.6	1.02	8.56	4445	0.14
Average	5.58	0.55	12.5	1.97	27.81	6008	0.19
Absolute s.d.	6.33	0.26	8.3	1.18	5.76	5430	0.17
Specimen 1, layer 3	8.77	0.60	9.2	5.26	38.79	9276	0.30
Specimen 2, layer 2	7.62	0.35	5.4	2.67	13.50	6646	0.21
Specimen 3, layer 3	4.62	0.67	13.4	3.10	43.24	4015	0.13
Specimen 4, layer 3	0.60	0.51	14.2	0.31	12.25	2514	0.08
Average (−2)	5.40	0.53	10.6	2.83	26.95	5613	0.18
Absolute s.d.	3.65	0.14	4.1	2.03	16.36	2980	0.10
Average (−2)	4.66	0.59	12.3	2.89	31.43	5268	0.17
Absolute s.d.	4.09	0.08	2.7	2.48	16.76	3551	0.12
Specimen 1, layer 4	4.51	0.80	12.3	3.61	26.62	1309	0.04
Specimen 2, layer 3	9.52	1.15	17.7	10.95	55.39	1038	0.03
Specimen 3, layer 4	3.20	0.11	2.2	0.35	4.88	2986	0.10
Specimen 4, layer 4	0.64	0.22	6.1	0.14	5.53	1689	0.05
Average	4.47	0.57	9.6	3.76	23.11	1756	0.06
Absolute s.d.	3.73	0.49	6.8	5.05	23.77	863	0.03
Average (−2)	2.78	0.38	6.9	1.37	12.34	1995	0.06
Absolute s.d.	1.97	0.37	5.1	1.95	12.37	879	0.03

TABLE VI—(*Continued*)

Specimen	Layer [V], mM	Layer volume ml	Per cent total volume	Vanadium concentration micromoles	Per cent total V	Layer cell count per cu mm	Per cent cells
Specimen 1, layer 5	14.83	0.10	1.5	1.48	10.91	197838	6.36
Specimen 2, layer 4	47.34	0.13	2.0	6.15	31.11	160900	5.15
Specimen 3, layer 5	12.94	0.07	1.4	0.91	12.69	101586	3.25
Specimen 4, layer 5	3.86	0.05	1.4	0.19	7.51	82273	2.63
Average	19.74	0.09	1.6	2.18	15.56	135874	4.35
Absolute s.d.	19.01	0.04	0.3	2.70	10.59	53625	1.72
Average (−2)	10.54	0.07	1.4	0.86	10.37	127532	4.08
Absolute s.d.	5.86	0.03	0.1	0.65	2.63	62417	2.00

binding and of the "chromogen" in the intact cell is not readily inferred from lysate data. Earlier (Webb, 1939) it had been suggested that the "chromogen" in vanadocytes is a bile pigment pyrrole, perhaps biliverdin (Lemberg and Legge, 1949). It is very likely, however, that the positive test for pyrroles resulted from the presence of hemoglobin in the lysate. Is it then likely that the spectrum of the intact vanadocyte is primarily due to a vanadium complex?

The electronic configuration of V(III) is [A] ($3d^2$), and attempts to provide spectral data on a $3d^2$ system in octahedral symmetry have therefore centered on V^{3+} ion in different compounds (Ballhausen, 1962). In aqueous solution $V(H_2O)_6^{3+}$ is blue, with maxima at 575 and 397 nm in the optical region. The bands have been successfully assigned to energy-level transitions based on ligand field theory. Shifts of the bands occur as a result of changes in ligand field strength; relative positions may also change when the symmetry of the field is changed. These effects can be accounted for satisfactorily in terms of the ligand field model (Cotton and Wilkinson, 1972; Ballhausen, 1962). None of the reported spectra resemble the whole blood spectrum shown in Figure 2. Consequently, the vanadium(III) complex is probably not involved to a significant extent in the optical absorption spectrum of *A. nigra's* yellow-green blood cells. The possibility that the absorption spectrum of the blood cells is due mainly to a wholly organic compound should then be considered; for example, comparison with chlorophyll and other hematin compounds may be made.

However, none of these compounds possess absorption spectra resembling that of the intact blood cells. Chlorophyll and the bile pigment biliverdin have optical spectra characterized by blue and red bands. The vanadocyte spectrum lacks the red band, and the peak at 320–325 nm lies too deeply in the uv to be a blue band. Absorption spectra of hemoglobin compounds (Lemberg and Legge, 1949) all show strong peaks at 400–450 nm which do not correlate with Figure 2. Thus, hematin-like complexes are probably neither responsible for the color of the blood cells, nor present to an extensive degree.

The closest meaningful spectral correlation between ascidian blood cells and a well defined compound that we have been able to find is with pteridines (Karrer, Manunta and Schwyzer, 1948; Gaill and Momzikoff, 1975). The relevant optical properties shown by pteridines are two to four uv bands (the spectra are strongly pH dependent due to tautomerism), and a maximum above 300 nm, but none above 400 nm (Blakeley, 1969). Xanthopterin, a pteridine obtained from *Microcosmus polymorphus,* has maxima at 281 and 373 nm, and exhibits fluorescence. The blood cell spectra (Fig. 2a and 2c) show bands in the uv and below 400 nm; no visible fluorescence of the whole blood cells is observed upon uv irradiation, however.

It is obvious that whole blood cells are complex mixtures for which a simple spectral assignment would not be anticipated (Swinehart, Biggs, Halko, and Schroeder, 1974). It is equally clear, however, that characteristics of the blood cell lysate, unless obtained and studied under conditions that are essentially intracellular, are misleading indicators of the chemical constitution of the blood cell. An organic compound dominates the absorption spectrum. This compound may be a pteridine, in which case the lack of fluorescence may be due to conditions within the cell.

This conclusion has interesting ramifications, for though *M. polymorphus* and *A. nigra* are in the class Ascidiacea, members of the family Ascidiidae possess vanadocytes, whereas members of the family Pyuridae (of which *M. polymorphus* is one) have ferrocytes (Endean, 1955; Smith, 1970) and are usually low in vanadium content (Swinehart *et al.*, 1974). The similarity in morphology, organic composition and structure of the blood cells, together with the similarity in appearance and ecology of the two genera suggest two successful evolutionary lines, in one of which (Ascidiidae) vanadium(III) or (IV) has the same function that iron(II) has in the other (Pyuridae) line.

The relative vanadium concentration of the layers, $5 > 2 > 3 > 4 > 1$, corresponds to the relative total cell count of the layers (Table VI). It would not be unusual for vanadium to be present in cells other than the green globular cells, and be of an oxidation state (pentavalent) that would not reduce OsO_4. This would imply that the relative vanadium concentrations of the layers are indicative of the overall cell counts, not the green globular cell frequencies. Data (Table VI) which correct for concentration effects and reflect the vanadium *contribution* by a particular layer toward the total vanadium in the blood (% Total V) show that each layer makes a significant contribution. This calculation suggests that vanadium is present in additional cell types.

It should be noted, however, that the cells in all of the Lymphoprep density centrifugation layers make up a very small part of a particular layer's total volume. It is possible that vanadium, in free or bound form, escapes into the soluble portions of the layers as a result of cell rupturing. Cell rupturing, if significant, would tend to erase the differences in vanadium concentration between layers, and produce a leveling effect.

Tunicates concentrate vanadium in the pentavalent state from sea water (Ladd, 1974; McLeod, Ladd, Kustin, and Toppen, 1975) and must obviously reduce the metal to either a trivalent or tetravalent oxidation state, since V(III) or V(IV) is present, depending on the tunicate order (Swinehart *et al.*, 1974). It is conceivable that less mature blood cells in a developmental scheme, such as the large smooth and small smooth cells, would possess pentavalent vanadium, which is suggested by the atomic absorption metal analyses of the Lymphoprep density centrifugation layers. During maturation, in the case of *Ascidia nigra,* the pentavalent vanadium would be reduced to the trivalent form, the presence of which is indicated by the lack of a vanadium signal in the epr spectrum and the OsO_4 staining. This hypothesis is similar to the actual mechanism of iron incorporation which occurs in human erythrocytes (Bloom and Fawcett, 1968; Harper, 1973). Coincident with this chemical reduction of vanadium, protein synthesis would be occurring in the cells, resulting in an increase in the density of the cells, which is reflected in the results of the Lymphoprep density centrifugation.

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SUMMARY

1. The blood plasma of *Ascidia nigra* has been characterized with regard to pH, salinity, and ultraviolet-visible absorption. Whole blood of *A. nigra* has been characterized with regard to cell count, cellular volume, hemoglobin (iron) content, vanadium content. Blood cell types have been examined, categorized, and differentially counted.

2. Epr and nmr studies prove that trivalent vanadium is present. Staining of whole blood slides with OsO_4 reagent identify the vanadium-carrying cells as being mainly the green globular blood cells. The observed uv-vis absorption spectrum does not correlate well with known vanadium(III) complex spectra.

3. Density separation of blood cells has been achieved; this result coupled with various cell morphologies suggests that different cells may represent different maturational stages in a developmental process.

4. Vanadium analyses of layerings of cells of different densities suggest that vanadium may be present in more than one type of blood cell, where, when not present as vanadium(III), it would be present in the diamagnetic, pentavalent oxidation state.

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THE EFFECT OF URETHAN ON HYDRA

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Although urethan (ethyl carbamate) is a common invertebrate anesthetic, very little is known about its mechanism of action. It is frequently used as a reversible anesthetic; that is, urethan may be applied to the animal which will be seen to relax; an experimental maneuver is performed; and the animal recovers and shows no long-term ill-effects after the urethan is washed away. However, in preliminary, but unreported, experiments with hydra (prior to Josephson and Macklin, 1969; and Macklin and Josephson, 1971), urethan was used to relax animals so as to facilitate experimental manipulation. It was discovered that the transepithelial potential of animals in urethan reversed; that is, the inside or gut of the animal is normally positive relative to the surrounding medium, but urethan would cause the inside to become negative with respect to the outside medium. Because of this, none of our previous experiments were conducted using urethan, and these preliminary results were ignored because of the difficulty they presented to the main experimental line. However, the effect of urethan is still of interest, not only because urethan is a common experimental drug but also because of this curious effect.

The literature is rather uniform in declaring that urethan acting on nervous tissue has *no* effect on transmembrane potentials and only has an effect on the conduction of action potentials, thus acting as an anesthetic (*e.g.*, Barker and Gainer, 1973; Il'inskii and Tertyshnik, 1960; Iwase, 1957; Kobayashi, 1963; Rudolph, 1953; Salmoiraghi and Weight, 1967; Sorokina, 1958; Thesleff, 1956). The same authors go on to say that the effect of urethan is a postsynaptic junctional effect. There is also a report by Wilbrandt and Schatzmann (1960) that whereas low electrolyte concentration media cause the cation permeability of red blood cells to increase, urethan partially blocks this effect.

The literature references quoted above typically used urethan in concentrations between 1 and 3% w/v (112 to 337 mM), which are similar to the concentrations used with coelenterates. Therefore, there seems to be a difference in the effects on nervous tissue and the effect on hydra epithelia. One clue to the possible consistency of effects is that urethan is claimed to stabilize the DC-potential across nerve membranes by an effect on sodium conductance; and sodium transport is felt to be responsible for the transepithelial potential reported in hydra (Macklin, 1967; Macklin and Josephson, 1971).

The experiments to be described were planned to gain additional insight into the mechanism of action of urethan on hydra.

Materials and Methods

Hydra were cultured in the manner of Loomis and Lenhoff (1956). Experimental animals were fed daily with newly hatched artemia. All experiments in

TABLE I

Experimental solutions used. All concentrations are expressed in mM/L.

Solution name	Na^+	K^+	Ca^{++}	Mg^{++}	Cl^-	$SO_4^=$	HCO_3^-	$EDTA^=$	Urethan
Culture	1.44		1.5		3.0		1.2	0.12	
Normal	1.5		1.5		3.0		1.5		
M	1.0	0.1	1.0	0.1	2.3		1.0		
Haynes	12.0	0.6	12.6	1.8	37.2	1.8	0.6		
Low calcium	1.44		0.15		0.3		1.2	0.12	
Urethan (2% w/v)	1.5		1.5		3.0		1.5		224

this series were conducted at 20 to 24° C with *Hydra oligactus* starved for 24 hours prior to use.

A number of solutions varying in ionic composition were used in the study (Table I). "Normal solution" which is the standard for comparison (Macklin and Josephson, 1971) differs from the culture solution primarily in the absence of EDTA. EDTA is included in the culture medium to remove heavy metal ions which can be toxic in small concentrations (Loomis and Lenhoff, 1956). All solutions were prepared in distilled water with reagent grade chemicals.

For the preparation of epidermal or gastrodermal isolates, a modification of an unpublished technique developed by M. Cyrlin and A. L. Burnett (Northwestern University, Evanston, Illinois) was used. The separation technique was checked by looking at whole mount preparations for the presence of gastrodermal pigment in epidermal pieces, and by looking for the characteristic mucous droplets of epithelial cells in gastrodermal pieces. Histological preparations confirmed the validity of the technique. Pieces which showed poor homogeneity of tissue on inspection were not used in experiments. In addition, for electrophysiological studies on regenerates, it was necessary that the piece form a cavity. In most cases, by 24 hours there was an identifiable cavity in the epidermal and gastrodermal isolates. The cell layer separation technique is summarized below.

To obtain gastrodermal explants, intact animals are placed in M solution (Muscatine, 1961) adjusted to pH 2.5 with 0.1 N HCl. After one minute in the acid M solution, the animals are transferred to Haynes solution (Haynes and Burnett, 1963). The epidermis contracts and may be removed by a combination of gentle swirling and manipulation with glass needles. Gastrodermal explants are then placed in fresh Haynes solution for 24 hours prior to use.

To obtain ectodermal explants, intact animals are placed in 10^{-5} M reduced glutathione in culture solution until a yawning response is obtained. Animals are everted by grasping the basal disc with number 5 jewelers forceps and pulling it through the mouth. The animals are transected at the subhypostomal level and placed in the low calcium solution. Agitation in this solution causes the gastrodermal layer to separate from the ectodermal layer. Ectodermal explants are placed in culture solution for 24 hours prior to use. Ectodermal explants all return to their normal orientation prior to the formation of a regenerate; *i.e.*, the usual outside of the regenerate faces the external medium. This mechanism of re-turning was previously reported by Macklin (1968).

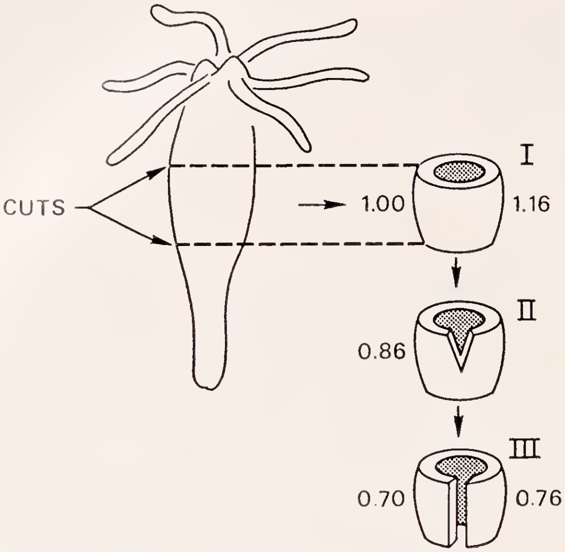

FIGURE 1. Method of preparing *Hydra* regenerates. The animal was cut twice as shown in the first diagram of the sequence and the mid-section of the animal (gastric region) was used. For the experiment in which the animal was cut longitudinally, configurations I, II and III identify the method of cutting. Configuration I is the regenerate as removed from the animal; in configuration II a longitudinal cut half-way down the regenerate was made; and in configuration III the longitudinal cut was completed. The numbers shown adjacent to the configurations give the relative length of the animal normalized to the length of configuration I in normal solution. The lengths on the left side are normalized lengths for maximal relaxation and those on the right side of the figure for maximal lengths in 2% w/v urethan. Data is from Table II.

To measure electrical potentials across the body wall, glass micropipettes filled with 3 M KCl with chlorided silver wire electrodes were used. Potentials were measured with the electrode tip in the gut cavity of the animal or regenerate using a high input impedance DC operational amplifier and displayed either on an oscilloscope or curvilinear chart recorder. Potentials were also read from a digital voltmeter. To verify representative resting potentials for each of the various conditions studied, preliminary testing established the time required for the potential to become stable. For a five minute test period, the resting potential was measured at 3, 4, and 5 minutes after a solution change, the average of these three values was used as a measure of the resting potential. Similarly, for a 20 minute test period, the resting potential was recorded 17, 18, 19, and 20 minutes after a solution change. All data reported are the result of the analysis of data from multiple animals for each separate experiment. Solution changes were such that we were assured of a greater than 99.9% exchange of solution. The experimental chamber used is the same one previously used in experiments reported in Macklin and Josephson (1971).

When the length of animal pieces were being measured, the pieces were examined immediately after preparation (Fig. 1) under a dissecting microscope with a micrometer eye piece. Each piece was watched and its length measured at its maximum value as the animal contracted and relaxed. The diagram in Figure 1 illustrates the various cuts made.

RESULTS

Relaxed length of hydra segments

Hydra have the usual arrangement of myotomes or muscle fibers as in other coelenterates; that is, an inner circular or helical layer and an outer longitudinal layer. This is the common pattern found in vertebrate tubular structures as well. Two effects are of interest here. The first is the relationship between the continuity of the inner helical layer of muscle fibers and the relaxed length of the animal; and the second is the effect of urethan on animal length. It is generally assumed that urethan is strictly an anesthetic and causes passive relaxation of the animal. It was this hypothesis that was being examined.

Figure 1 shows the sequence of cuts that were made in this series of experiments. An animal had its hypostome and peduncle surgically removed and was then immediately examined under a dissecting microscope. Lengths were measured as follows. In the first series of experiments the length of the excised segment (Fig. 1, configuration I) was observed and the maximum relaxed length was recorded. The segment was cut half-way down the length of the cylinder (II) and once again the length of the maximum relaxed length of the segment was recorded. The cut was then extended all the way down (III), the animal was continually observed, and its maximum length recorded. This final segment (configuration III) was then placed in 2% w/v urethan and its maximum length was once again recorded. The summarized results are recorded as Experiment 1 in Table II. In a second series, configuration I segments were not cut longitudinally but had their maximum lengths measured in the normal solution and in the urethan solution (Experiment 2, Table II).

Normalized results shown in Figure 1 summarize the data from 34 separate animals with their lengths all normalized to the length of the animal in its maxi-

TABLE II

The mean length of fully relaxed Hydra oligactus *pieces is contrasted for intact gastric segments (configuration I) and the same segments, cut as shown in Figure 1, in culture medium and in culture medium with 2% w/v urethan added. The values of* P *shown are for Student's* t-*test for paired data for a comparison of each length with the immediately preceeding length in the table.*

	Configuration	Urethan	Length ± s.e.	*P*
Experiment 1	I	No	2.5 ± 0.2 mm	
(n = 22)	II	No	2.1 ± 0.1 mm	<0.001
	III	No	1.6 ± 0.1 mm	<0.001
	III	Yes	1.9 ± 0.1 mm	<0.01
Experiment 2	I	No	2.6 ± 0.2 mm	
(n = 12)	I	Yes	3.0 ± 0.2 mm	<0.001

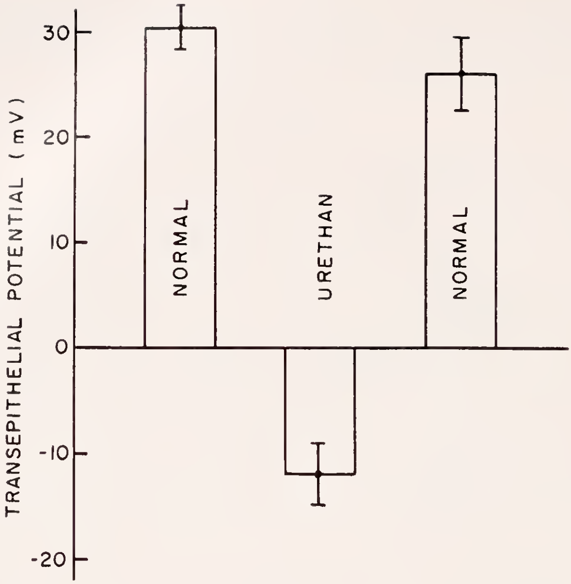

FIGURE 2. Effect of 2% w/v urethan on transepithelial potential in *Hydra oligactus*. Hydra were held on the special glass holder and potentials measured as described in Josephson and Macklin (1971). Normal solution was placed on the outside of the animal, exchanged for the 2% w/v urethan and then exchanged for normal in sequence. The mean ± standard error for seven animals are shown. Using Student's *t*-test, the difference between the transepithelial potential in normal and urethan solutions is significantly different ($P < 0.001$). The two values in normal solution are not significantly different ($P > 0.2$). Animals were in each test solution for twenty minutes and readings were taken every minute for the last four minutes. The method is described in the text.

mum relaxed length in configuration I. Note that the maximum length decreases as continuity of the circular muscle layer is destroyed. The length of the longitudinally cut animal is only 70% of the maximum length that the animal can achieve without this cut. In addition, once the animal is placed in urethan, whether it is a segment with the circular muscles intact (I), or if the continuity of the circular muscles are completely destroyed (III), there is an increase in length in both cases. For the intact circular pattern, there is a 16% increase due to urethan, and for the severed pattern it is a 9% increase.

Two points emerge from these observations. First, complete relaxation or elongation of hydra is the result of at least two events: the relaxation of the longitudinal muscle layer, and the active contraction of the inner circular muscles. The active contraction is apparent because once the continuity of the inner muscle layer is severed, the maximum relaxed length of the animal is reduced (Experiment 1, Table II). The results suggest that 70% of the lengthening of the relaxed animal is due to the relaxation of the outer layer and an additional 30% lengthening is due to a contraction of the inner muscle layer. Secondly urethan is not solely an outer longitudinal muscle layer anesthetic as generally assumed. That is, it not only has a relaxing effect, but it appears to have an active effect causing the inner muscle layer to contract. Alternatively, we could assume that the outer longitudinal muscles do not fully relax unless they are placed in urethan. However, the fact that severing the circular muscles causes a statistically significant reduction in the lengthening ability of the animal points to the conclusion that the circular muscles are partly responsible for the length of the fully relaxed animal.

Effect of urethan on transepithelial potentials

These results are the most interesting and least explicable in this series of experiments. Without exception, in all of the experimental manipulations, urethan causes the normal positive transepithelial potential in hydra to become negative (Fig. 2). Also, urethan causes the contraction pulses (CP's) to diminish in size (Fig. 3). In other recordings, occasional biphasic and inverted contraction pulses were seen. The presence of a biphasic CP is uncommon in our experience.

Since several different manipulations were performed, they will be discussed in turn. The effect of urethan on intact hydra was determined using the special glass holder previously described by Josephson and Macklin (1969); and the effect of urethan on gastric region regenerates and epidermal and gastrodermal cell layer isolates was determined using micropipette impalement into the gut cavity. In all experimental situations, the same experimental protocol was followed. The animal was first placed in normal solution for a time period sufficient for the transepithelial resting position to stabilize; the bathing solution was changed to the urethan solution, the animal was held in that solution for 5 to 20 minutes, and then the animal was returned to the normal solution. The potential in the urethan solution differed significantly from the potential in the normal solution—both the initial and the final normal solution changes—for all animal forms tested except for gastrodermal layer isolates.

When the transepithelial resting potential was measured for intact hydra (Figs. 2 and 3) or for regenerates (Fig. 4), the same result was observed. Urethan

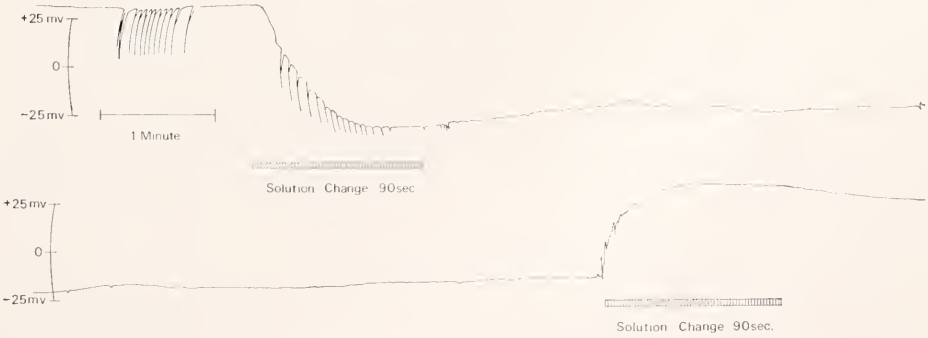

FIGURE 3. Tracing of recording from a curvilinear recorder showing the transition from normal solution to urethan and back to normal solution. The original recording was traced for clarity. Note in particular how the contraction pulses (CP's) decline in the urethan solution and the rapidity of the change in potential when solutions are changed.

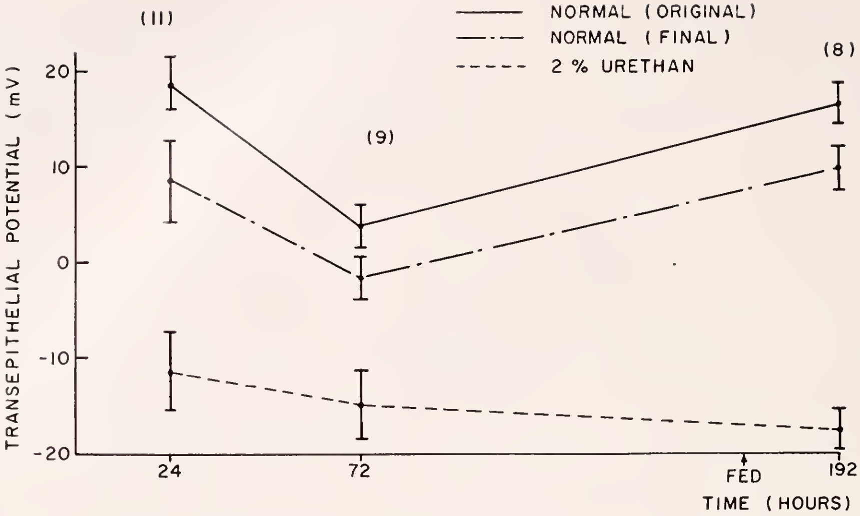

FIGURE 4. Effect of urethan on transepithelial potential in *Hydra* regenerates. For this experiment glass micropipettes were used to measure the transepithelial potential in hydra regenerates. For the sequence shown, eleven animals were used; however, three animals were lost so that the value for N differs during the sequence. From this data, it can be seen that the transepithelial potential is significantly less in the urethan solution than in the normal solution for each test time ($P < 0.001$). Also, it is interesting to note that there is initially a decrease in transepithelial potential; and following feeding the potentials increase. The experiment was conducted by first placing the animal in normal solution, then in 2% w/v urethan, and then back into the normal solution. Animals were held in each solution change for five minutes and the values used for determining the potential were recorded every minute for the last three minutes using the method described in the text.

causes the resting potential to drop significantly ($P < 0.001$) and to reverse polarity. The gut potential which is normally positive with respect to the medium becomes negative. CP's also always diminish in size and may become inverted or biphasic. The result does not appear to be analogous to previously reported effects of osmotic pressure (Macklin and Josephson, 1971). This is based on the previous observation that an osmotic pressure of 67.5 mOsmol or greater produced irreversible short term changes in transepithelial resting potential, whereas urethan does not ($P > 0.2$). It is therefore hypothesized that increasing osmotic pressure in the bathing medium with sucrose, a nonpermeate solute, has an entirely different effect on the ion transporting system from increasing the osmotic pressure with urethan, a highly permeate solute.

To localize the effect of urethan in the hydra body wall, a second series of experiments were conducted in which cell layer isolates were compared with regenerates and intact animals (Table III). All four animal configurations were tested under the same conditions using micropipette electrodes inserted through the body wall into the gut cavity. Except for the gastrodermal layer isolate, there is a significant ($P < 0.001$) reduction in transepithelial resting potential attributable to the urethan. Urethan does not effect the transepithelial potential of gastrodermal cell layer isolates. Only cell layer isolates with an easily identifiable cavity 24 hours after preparation were used in these experiments.

Discussion

All the results point to the conclusion that there are at least two effects of urethan on hydra. The effect on electrical activity can be further subdivided into a change in transepithelial potential and an effect on contraction pulses. The second effect is on the contraction of the gastrodermal muscle layer.

Previous results (Macklin and Josephson, 1969) demonstrate that the normal resting potential in hydra is related to a flow of sodium ions from the culture medium to the gut—the sodium being transported by an electrogenic mechanism. It has been further hypothesized (Macklin, 1967) that the gastrodermal cell layer is also capable of actively transporting sodium in the opposite direction; but there is an adaptational effect of the cell layers based on their location in the animal such that in normal free-living hydra the net flow of sodium is inward. This is clearly the required direction for net ion transport for osmotic equilibrium. However, urethan causes the potential difference to reverse; that is, the outside becomes negative relative to the inside of the animal. This further supports the existence of two transport systems. The usual net flow of sodium ions is from the medium into the animal tissue, therefore it is possible that urethan abolishes the flow of sodium from the medium to the epithelial cell layer. What is observed in the presence of urethan is only the sodium current from the gut cavity into the animal tissue. Since animal tissue and the gut have a higher sodium concentration than the medium (Steinbach, 1963), simply abolishing active inward flow would result in a reversal of the transepithelial potential because the Nernst potential would

Table III

Effect of urethan on mean ± standard error of transepithelial potential in Hydra oligactus. *For this comparison all animals were impaled with glass micropipettes and sequentially placed in normal, urethan, and normal solutions. Recording techniques are described in the text. The transepithelial potentials in urethan statistically differ from the initial and final potentials in the normal solution for the first three experiments (Student's* t*-test,* $P < 0.001$*). However, when only the gastrodermal layer is present, urethan does not effect the transepithelial potential (*$P > 0.2$ *for the first two values and* $P > 0.8$ *for the second two values). Animals were in each solution change for five minutes; measurements were recorded each minute for the last three minutes.*

Animal configuration	Solution		
	Normal (initial)	2% Urethan	Normal (final)
Intact animal (n = 11)	20.8 ± 1.1 mV	−8.0 ± 1.6 mV	16.3 ± 2.0 mV
24 hour regenerate (n = 11)	18.8 ± 2.7	−11.3 ± 4.0	8.5 ± 4.2
24 hour epidermal layer isolate (n = 10)	11.2 ± 2.2	−3.5 ± 3.1	12.9 ± 3.1
24 hour gastrodermal layer isolate (n = 10)	1.0 ± 0.6	0.1 ± 0.5	0.1 ± 0.4

remain. Also, when isolated cell layers were used (Table III), it was noted that for the isolated epithelial layer the potential reversed, whereas for the gastrodermal cell layer there was essentially no effect on transepithelial potential. This implies that even if there is a bidirectional sodium current in the intact animal with the gastrodermal cell layer pumping in opposition to the epidermal cell layer, when the gastrodermal layer is removed there should only be a unidirectional sodium current in the isolated epithelial cell layer. Therefore, a more consistent conclusion based on the data is that urethan causes the epithelial cell layer to become leaky so that there is then a net flow of sodium ions from the tissue into the external medium. The negative transepithelial potential then is a measure of this passive flow. This is consistent with the observation that when the animal is transferred from urethan solution back to normal solution the final potential is slightly, but not significantly less than the initial potential. This is similar to previous results (Macklin and Josephson, 1971) with animals transferred from solutions containing calcium to solutions without calcium, where there was apparently structural damage due to the short term absence of calcium in the medium. An alternative explanation for the transepithelial potential reversal in the presence of urethan is that urethan inhibits the electrogenic transport system. The normal diffusion of sodium out of the epithelial cells due to the electrochemical gradient would then account for the apparent damage after removal of urethan from the bathing medium.

The effect of urethan on the contraction pulses is likewise of interest. This effect has been previously noted in other experimental preparations (Macklin and Josephson, 1971) where biphasic contraction pulses and the inversion of contraction pulses were seen when calcium was removed from the gut of the animal. It was concluded that the contraction pulse was a calcium spike. The action of urethan thus may be on the calcium mechanism. However, it is more reasonable to conclude that urethan causes a transient transport defect, or damage to the tissue in much the same way that absence of calcium caused damage. This conclusion is further supported by unpublished experiments with animals which were starved for three weeks and by experiments with animals which were exposed to nitrogen mustard. In the experiments with nitrogen mustard and in the experiments with long-term starvation, the same result was obtained; that is, contraction pulses consistently became very small, typically became biphasic and frequently were seen to be positive going rather than the usual negative going spikes. Consequently, the effect on contraction pulses observed with urethan, calcium, and nitrogen mustard, and those due to starvation are probably all the same effect. It is probably a membrane damage effect and not a specific ion effect. The removal of calcium typically increases membrane permeability and we therefore conclude that these results are due to a generalized increase in membrane permeability leading to a diminution of contraction pulses and their biphasic nature. Also, since the contraction pulse is considered to be generated on the inward facing cell layer, it is immune to external medium ionic concentrations unless the solute is freely permeable. This is true for urethan, probably true for nitrogen mustard over a long-term period, and is consistent with the finding that calcium had to be removed from the gut before the effect was seen, whereas short-term calcium removal from the external medium did not produce this effect. The effect on CP's in all experimental manipulations is reversible.

The effect of urethan on action potentials in nervous tissue which has been reported extensively (Barker and Gainer, 1973; Il'inskii and Tertyshnik, 1960; Iwase, 1957; Kobayashi, 1963; Rudolph, 1963; Salmoiraghi and Weight, 1967; Sorokina, 1958; Thesleff, 1956) is only partly consistent with the results with hydra. That is, whereas action potentials disappeared when urethan in concentrations of 1 to 3% is used, membrane potentials stabilize in the other preparations. However, transepithelial potentials in hydra do not stabilize but change significantly.

Urethan has a more extensive effect on hydra than the relaxation of myotomes, which is the typical casual observation of most investigators. The experiments summarized in Table II clearly show that urethan causes the inner muscle cell layer in the gastrodermal layer to actively contract. This was demonstrated two ways: when the regenerate is intact, it lengthens in the presence of urethan over its maximal relaxed length without urethan; when a longitudinal cut is made, urethan is still able to cause an increase in length of the cut segment. However, the cut segment cannot achieve the length of the uncut segment (Experiment 1, Table II). This strongly suggests that for the animal to fully relax, the internal muscle layer must be intact. Therefore, lengthening or relaxation for hydra is probably a two-step process involving both contraction of the inner muscle layer and relaxation of the outer longitudinal muscle layer. The reason that corresponding electrical activity is not recorded for the elongation of the animal has never been clear, although it is apparent that the inner muscle cell layer is, in fact, involved in animal elongation.

In summary, urethan not only has a profound effect on the transepithelial potential and therefore the electrogenic sodium transport mechanism of hydra, but it also has an effect on the gastrodermal myotomes causing active contraction of that muscle layer.

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SUMMARY

1. The relaxed length of hydra is increased in the presence of urethan. This suggests a direct effect of urethan on the inner circular myotome layer.

2. Urethan causes the normal transepithelial resting potential to reverse such that the gut becomes negative relative to the external medium.

3. Urethan also causes contraction pulses to become reduced in magnitude.

4. It is suggested that urethan has both a direct effect on the hydra myotomes and on the sodium transport mechanism.

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THE IONIC ENVIRONMENT OF HEMOCYANIN IN *LIMULUS POLYPHEMUS*

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The extracellular respiratory pigments found in invertebrates are, in general, highly sensitive to their ionic environment in the blood. The ion dependence of molecular structure and *in vitro* oxygen binding of the hemocyanins has been investigated intensively. Small changes in H^+ or inorganic salt concentrations, for example, bring about large changes in the equilibrium between native molluscan hemocyanin and its constituent subunits (DePhillips, Nickerson and Van Holde, 1970). These structural changes are accompanied by shifts in oxygen affinity and the cooperativity of oxygen binding.

The effective ions that have received the most attention are H^+ and the divalent cations Ca^{+2} and Mg^{+2}. Interestingly, H^+ and the ions of inorganic salts have opposite effects on *in vitro* oxygenation. If an increase in H^+ concentration reduces oxygen affinity (*e.g.*, the extracellular heme proteins and crustacean hemocyanins), an increase in salts raises oxygen affinity (Antonini, Rossi-Fanelli and Caputo, 1962; Chantler, Harris and Bannister, 1973; Everaarts and Weber, 1974). Conversely, if an increase in H^+ raises oxygen affinity (*e.g.*, gastropod and xiphosuran hemocyanins), then an increase in inorganic salts lowers oxygen affinity (DePhillips *et al.*, 1970; Sullivan, Bonaventura and Bonaventura, 1974). Although the divalent cations in blood change very little with acclimation salinity, the physiological changes appear to be great enough to induce detectable shifts of the *in vitro* oxygen affinity of hemocyanin from the portunid crab *Carcinus maenas* (Truchot, 1973, 1975). In addition, more variable ions such as Na^+ and Cl^- modify the oxygen affinity of other extracellular respiratory pigments, such as annelid hemoglobins and xiphosuran hemocyanins (Everaarts and Weber, 1974; Sullivan *et al.*, 1974).

The salt dependence of oxygen carrying pigments suggests that the oxygen transport system must be influenced by environmental salinity in estuarine species that are not perfect regulators of the ion concentrations in their body fluids. In a study of oxygen transport in the portunid crab *Callinectes sapidus*, it was shown that *in vivo* oxygenation of hemocyanin remains essentially unchanged even when blood salts are diluted, because the salt effect is opposed by a concomitant elevation of blood pH (Mangum and Weiland, 1975; Weiland and Mangum, 1975). The pH change at low salinity is believed to result from the increased production of ammonia, which binds H^+, raises pH and opposes the effect of salt depletion on hemocyanin oxygenation (Weiland and Mangum, 1975). The increased ammonia, which may be produced in the catabolism of free amino acids as intracellular fluids re-equilibrate to the dilute blood (Gerard and Gilles, 1972), also plays a role in salt absorption at the gill. In microsomal preparations, NH_4^+ serves equally well as K^+ as the counterion for activity of the transport enzyme that moves Na^+ across the gill membrane (Towle and Harris, 1976). When *C. sapidus* osmotically conforms to a salinity reduction, no change in NH_4^+ output occurs, but there is a large increase when the medium is diluted further to a salinity at which the crab actively maintains a hyperosmotic blood (Mangum, Silverthorn, Harris, Towle and Krall, 1976).

Thus the oxygen transport system in *C. sapidus* is highly adapted to life in an unstable ionic environment. The mechanism may be categorized as *enantiostatic,* in the sense that the standing condition, a highly integrated response of the respiratory, osmoregulatory and excretory systems, results from opposite and counterbalancing ionic effects on the respiratory pigment (Mangum, 1976). There are ample reasons to expect that these findings are not general among estuarine animals and that the enantiostatic balance observed in portunid crabs is but one of many possible adaptations. Therefore we have investigated the influence of acclimation salinity on oxygen transport in the xiphosuran *Limulus polyphemus* (Linnaeus), which differs in several important respects from decapod crustaceans. First, its hemocyanin has different ionic sensitivities; the Bohr shift is negative and the addition of salt lowers oxygen affinity. The influence of salts on oxygen affinity is due in large part to Cl^- and not only to divalent cations (Sullivan *et al.*, 1974). Stripping the blood of all salts increases oxygen affinity from 6.8 to 1.6 torr at 20° C and pH 7.5, and the addition of NaCl to the stripped preparation induces a logarithmic decrease to about 3.8 torr at 4 M NaCl. The chloride effect on whole blood has been quantitatively explained by the sensitivity of two of the five hemocyanin components separated by ion exchange chromatography. Second, salinity-induced changes in blood NaCl and Ca^{+2} concentrations in *Limulus* are more than twice as great (Robertson, 1970). Thirdly, less than 10% of the change in intracellular osmolality in *Limulus* is due to free amino acids (Bricteux-Grégoire, Dûchateau-Bosson, Jeuniaux and Florkin, 1966; Robertson, 1970), while the comparable figure for *C. sapidus* is 70% (Gerard and Gilles, 1972). Last, horseshoe crabs enter the estuary only in the late spring and summer, and they do not penetrate very far into the deeper and more dilute regions.

MATERIALS AND METHODS

Animals were obtained from fishermen or by personal collection in high salinity waters (31–33‰) near Wachapreague on the Eastern Shore of Virginia, where the work was conducted. They were maintained in running sea water until a few days prior to experimentation. Sampling was completed within one week of collection, so that no animal was in an extreme state of starvation and hemocyanin depletion.

Animals were exposed to low salinity in aerated containers for two days prior to sampling. A second set of samples was taken after five or seven days at 15.4–16.5‰ salinity, but no significant differences were noted. Dilutions were prepared with distilled water, and the salinities reported were calculated from measurements of the chloride ion (Buchler-Cotlove chloridometer). Acclimation to 8.8–9.4‰ was carried out in two steps, with 24 hr at an intermediate salinity.

Measurements on whole animals

The rate of oxygen consumption was calculated from a recorder trace of oxygen depletion in a closed container. Oxygen concentration was monitored continuously with a Yellow Springs Instrument Co. Model 5420 polarographic electrode and 180 recorder. Because of the size of the respirometer (12 l, including a tubular extension for the telson), additional stirring was provided at each of two sides, with large magnetic stirrers.

Total outputs of ammonia and acids were calculated from measurements of the two variables in 45 l sea water, made before and after confining an animal for 70–120 min. Net acid output was estimated from titration curves describing the change in pH after the addition of 0.1 N HCl to water at each salinity; the water was aerated for two hr prior to each measurement to equilibrate it to atmospheric CO_2.

The heartbeat of intact, unrestrained animals was recorded with an impedance pneumograph (E and M Instr. Co.). Electrodes were inserted on either side of the heart through small (1 mm diameter) holes drilled in the dorsal prosoma, and the holes sealed with dental wax (Surgident). Animals survived the operation for as long as five months.

Blood and urine sampling

Large samples (2–3 ml) of blood were taken from the pericardial sinus (postbranchial) or the junction of the mesosoma and telson (prebranchial) into iced syringes, within 15 sec of removing an animal from water. One aliquot was immediately injected into the chamber of a Radiometer Corp. BMS1 blood gas analyzer for PO_2 measurement. The remainder was centrifuged at high speed (Sorvall SS-1) to express serum from the clot, and further subdivided for the other measurements.

Urine was allowed to drip into small snap cap vials through a length of polyethylene (No. 10) tubing inserted into the opening of the coxal gland. Most of the measurements on urine were made immediately after sampling. However, the measurements of total solids, Na^+ and a second measurement of Cl^- were made on samples that had been stored for one to two weeks. Evaporation must have occurred in some of the vials, as indicated by comparing the results for total solids with those for osmolality, and by comparing the two sets of Cl^- data. These samples were used only for the calculations of the ion ratios, and the Na^+ concentrations were predicted from the first set of Cl^- values.

Because of an interest in the respiratory role of the excretory organ, it was important to obtain urine samples from animals that were not in a state of respiratory acidosis. And yet catheters inserted into the opening of the coxal glands did not remain in place when the animals were allowed to swim freely in water. Therefore the measurements were made on urine samples obtained within five to ten min of removing the animal from water. Blood samples taken after much longer air exposure indicate that significant acidosis occurs in 60 min ($\Delta pH = -0.19$ to -0.24), but that change would explain only a small fraction of the results reported below. In addition, several samples were obtained from animals submerged in small containers where they could not crawl or swim; the results for these samples do not show perceptible trends and they are statistically homogeneous ($P > .05$) with the others. There is no significant change in the ammonia concentration of urine after one hr air exposure ($\Delta NH_4^+ = -0.20$ to 0.012 mM/l).

Measurements on blood and urine samples

pH was measured with a liquid junction capillary electrode (Radiometer Corp.). Ammonia concentration was determined by the phenol hypochlorite method (Solórzano, 1969), using 0.5 ml samples. At high concentrations, we found it necessary to dilute the samples with distilled water and to allow two hours for completion of the reaction. In these cases, the background concentration of the ammonia in H_2O was subtracted from the results. Otherwise, the procedure was unchanged.

Sodium concentration was measured with an EEL flame photometer (Oglesby, 1970), and chloride with a Buchler-Cotlove chloridometer. Osmolality was determined with an Osmette freezing point osmometer, and total solids with an Atago refractometer.

The absorbance of blood was measured at 340 nm (Beckman DK-2 spectrophotometer) after dilution of the sample with distilled water (1:39). The concentration of hemocyanin was computed from the molecular extinction coefficient given by Nickerson and van Holde (1971). Oxygen carrying capacity at 159 torr was calculated from these results on the assumption that 0.17% of the total weight of the hemocyanin molecule is copper, and that Cu combines with molecular oxygen in the ratio 2:1 (van Holde and van Bruggen, 1971). A test of this procedure, made by comparing the results with those of more time-consuming but direct measurements (Mangum, Freadman and Johansen, 1975), indicates that it is equally accurate.

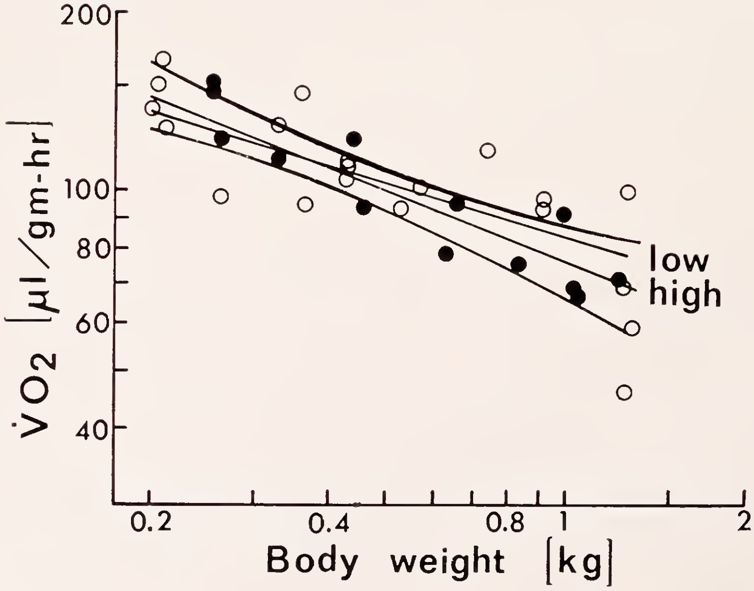

FIGURE 1. The rate of oxygen consumption ($\dot{V}O_2$) in *Limulus polyphemus* acclimated to 32 (closed circles) and 8 (open circles) ‰ salinity and 22° C. The 95% confidence interval encloses the regression line describing the combined data. The slope of that line is −0.39 (± 0.066) and the correlation coefficient (*r*) is 0.935.

Estimates of hemocyanin oxygenation

The *in vivo* oxygenation of hemocyanin was estimated by extrapolation to *in vitro* oxygen equilibrium curves. These curves were calculated for a temperature of 24° C and each relevant combination of H^+ and NaCl, from data shown by Sullivan *et al.* (1974) and Bonaventura, Sullivan, Bonaventura and Bourne (1974), further details of which were kindly communicated by the authors. The difference between stripped and unstripped blood was assumed to be due to logarithmic increases in NaCl, as shown by Sullivan *et al.* (1974), and also to other inorganic salts, as present in the ratios reported by Robertson (1970). The temperature dependence of oxygen binding was assumed to be conventional ($\Delta H = -13$ kcal/mole) as suggested by the results of Redfield and Ingalls (1933).

The quantitative effects of salts on the cooperativity of oxygen binding (the Hill constant n) are not known, but they are believed to be due to changes in divalent cations as well as NaCl. The physiological changes in Ca^{+2} reported by Robertson (1970) would cause a large change in the cooperativity of crustacean hemocyanins (Larimer and Riggs, 1964). For the present purposes, a linear reduction in the values of n reported by Redfield and Ingalls (1933) and by Sullivan *et al.* (1974) was assumed to occur with changes in blood Ca^{+2} and Mg^{+2} at low salinity.

RESULTS

Body size and salinity tolerance

Despite the precaution of a stepwise dilution, the larger animals in the sample died at 8.8–9.4‰ salinity. After two such losses of the material available, the larger animals were saved for experimentation at a less extreme dilution (15.4–16.6‰). The result is an unfortunate discrepancy in body size: the control group is representative, the group at 15.4–16.6‰ consists of atypically large animals (> 4 kg), and that at 8.8–9.4‰ of small animals (0.3–1.3 kg). Many of the measurements were performed as paired observations on the same individuals, and therefore the results are unaffected. However, the bias does affect several of the respiratory calculations, as discussed below.

This observation suggests that the surprisingly wide range of salinity tolerance reported by Robertson (1970), who studied only animals weighing less than 0.7 kg and who noted a size trend even within that sample, is misleading. In the Chesapeake Bay region horseshoe crabs are rarely found in waters of less than 18‰ (Wass, 1972 and personal communication). On one occasion we have personally observed animals in more dilute waters, 13–16‰ in Long Island Sound, but they were very small. Although Robertson (1970) cites several reports that would suggest otherwise, the observations made by the present authors and those of more experienced observers indicate that the species is largely confined to the polyhaline regions of estuaries, and that only the very young penetrate into mesohaline waters.

Oxygen consumption

Regression lines describing the data (Fig. 1) for oxygen consumption as a function of body weight at high (N = 13) and low (N = 21) salinities are not

TABLE I

Measured respiratory, osmotic and ionic parameters in blood and urine of Limulus polyphemus *held in waters of different salinity. Mean (± s.e.). Number of animals given in parentheses; number of observations given in brackets. High* O_2 *=* $\geq$ *140 torr; reduced* O_2 *= 98–105 torr; 22–24° C.*

	Salinity (‰)		
	32–33	15.4–16.5	8.8–9.4
1. Prosomal width (cm)	23.3 (±1.5) (10)	>30 (6)	18.4 (±1.1) (8)
2. Wet weight (kg)	1.882 (±0.371) (10)	>4.0 (6)	0.824 (±0.142) (8)
3. Sex			
Male	(3)	(3)	(3)
Female	(7)	(3)	(5)
4. Postbranchial blood PO_2 (torr) high O_2	75 (±3) (6)	62 (±8) (5)	66 (±3) (4)
reduced O_2	62 (4)	50 (5)	50 (4)
5. Prebranchial blood PO_2 (torr), high O_2	14.0 (±0.5) (9)	8.0 (±0.7) (6)	11.8 (±1.1) (6)
reduced O_2	8.0 (±1.3) (45)		5.8 (±0.7) (6)
6. Blood hemocyanin concentration (gm/100 ml)	9.08 (±1.08) (9) [16]	12.14 (±1.97) (5) [10]	9.87 (±1.58) (6) [10]
7. Prebranchial blood pH, high O_2	7.495 (±0.007) (9) [46]	7.522 (±0.010) (6) [17]	7.537 (±0.010) (6) [35]
Blood H^+ concentration/water H^+ concentration	1.94 (±0.06)	1.52 (±0.05)	1.12 (±0.08)
8. Urine pH	6.849 (±0.049) (6) [18]	7.216 (±0.026) (3) [6]	7.772 (±0.036) (5) [10]
Urine H^+ concentration/blood H^+ concentration	3.59 (±0.34)	0.98 (±0.25)	0.46 (±0.06)
9. Blood NH_4^+ concentration (mM/l)	0.092 (±0.003) (9) [42]	0.051 (±0.003) (6) [7]	0.048 (±0.006) (6) [21]
Blood NH_4^+ concentration/water NH_4^+ concentration	8.4 (±2.1)	11.3 (±1.4)	9.6 (±1.6)
10. Urine NH_4^+ concentration (mM/l)	0.650 (±0.085) (6) [8]	0.139 (±0.041) (3) [7]	0.052 (±0.009) (4) [5]
Urine NH_4^+ concentration/blood NH_4^+ concentration	8.35 (±1.12)	1.87 (±0.43)	1.57 (±0.31)

TABLE I—*Continued*

	Salinity ($^0/_{00}$)		
	32–33	15.4–16.5	8.8–9.4
11. Blood osmolality (mOsmol/l)	935 (±5) (10) [19]	540 (±15) (6) [13]	491 (±10) (8) [12]
Osmotic difference across gill membrane	6 (±2)	70 (±18)	209 (±13)
12. Urine osmolality (mOsmol/l)	948 (±5) (6)	562 (1)	410 (±12) (4)
Osmotic difference across coxal gland membrane	1 (±9)	25	68 (±20)
13. Total solids in urine (gm/100 ml)	3.0 (±0.1) (4) [7]	2.0 (±0.2) (4) [4]	1.4 (±0.1) (2) [4]
14. Blood Na^+ concentration (meq/l)	459 (±9) (10) [15]	272 (±12) (6) [11]	266 (±11) (7) [10]
Blood Na^+ concentration/water Na^+ concentration	0.95 (±0.01)	1.09 (±0.04)	2.17 (±0.09)
15. Urine Na^+ concentration (meq/l)	504 (±16)	282*	211*
Urine Na^+ concentration/blood Na^+ concentration	1.07 (±0.02)	0.97*	0.79*
16. Blood Cl^- concentration (meq/l)	488 (±11) (10) [23]	277 (±6) (6) [22]	226 (±13) (8) [22]
Blood Cl^- concentration/water Cl^- concentration	0.92 (±0.02)	1.10 (±0.04)	1.53 (±0.08)
17. Urine Cl^- concentration (meq/l)	517 (±16) (9) [21]	297 (±12) (3) [6]	229 (±7) (5) [7]
Urine Cl^- concentration/blood Cl^- concentration	1.06 (±0.02)	0.99 (±0.02)	0.88 (±0.04)

* See text.

significantly different from one another ($P = 0.35$, F test). This conclusion is supported by two sets of paired observations of oxygen consumption in the same individuals at high and low salinity, which are also homogeneous. If the salt effect on the oxygenation of hemocyanin is great enough to perceptibly influence its role in oxygen transport, then other respiratory compensations must occur.

Oxygen consumption does not decrease significantly ($P > 0.05$ according to

Student's *t* test) from the values shown in Figure 1 when the water PO_2 is lowered from 159 to about 80 torr. This finding agrees with the previous report of Johansen and Petersen (1975).

Effect of acclimation salinity on osmotic and ionic constituents of blood and urine

The results (Table I) on blood NaCl and osmotic concentrations are essentially the same as those reported earlier by Robertson (1970), indicating that body size does not influence the osmotic and ionic steady state, even though it is related to salinity tolerance. No sexual differences were noted in either his experiments or ours. At high salinity the blood of horseshoe crabs is slightly hypoionic and hyperosmotic to the medium. The hypoionic condition of blood appears to be maintained, at least in part, by the excretory organ, as in crustaceans. There is no evidence, however, that blood hyperosmoticity results from the reabsorption of osmotically active substances in primary urine.

At 8.8–9.4‰ the reduction of osmotically active substances in blood is opposed by the output of a dilute urine, and the difference between blood and urine is largely explained by the reabsorption of NaCl from the urine. Despite the regulatory response, the reduction of blood NaCl at low salinity is quite great. The decrease in Cl^-, the ion responsible for much of the change in hemocyanin oxygen affinity when salts are removed from blood (Sullivan *et al.,* 1974), is greatest when the acclimation salinity is lowered to 15.4–16.5‰. Further change at 8.8–9.4‰ is disproportionately small.

The ammonia concentration in the blood of horseshoe crabs at 32–33‰ is high relative to that in decapod crustaceans (Gerard and Gilles, 1972; Siebers, Lucu, Sperling and Eberlein, 1972). Unlike the portunid crabs, the level of ammonia in blood decreases at low salinity, a finding which is consistent with the evidence that reduction of the free amino acid pool is not a major mechanism of intracellular osmotic adjustment (Bricteux-Grégoire *et al.,* 1966; Robertson, 1970). The concentration of ammonia in urine decreases at low salinity to a level that, at 8.8–9.4‰, is no longer significantly higher than blood. At the same time, the total output of ammonia and net output of acids also decrease. Ammonia output in six animals decreased from 0.33 (± 0.10 s.e.) mM/kg-hr at 32.1‰ to 0.18 (± 0.03) mM/kg-hr at 15.4–16.5‰; net acid output decreased from 0.78 (± 0.08) mM/kg-hr at 32.1 to 0.20 (± 0.02) mM/kg-hr at 15.4–16.5‰. These results suggest that NH_4^+ in xiphosurans does not play an important role in salt absorption, in contrast with its importance in the blue crab *Callinectes sapidus,* and they do not suggest H^+ secretion. Due to the methodological difficulties of distinguishing between the movement of H^+ *per se* and changes in the output of a base or some other acid, this result is not a conclusive finding of lower H^+ output at low salinity, however. (The properties of the transport enzyme in the *Limulus* gill are presently under investigation by D. W. Towle.)

Thus the reduction of blood NaCl is not accompanied by the large increase in pH that occurs in portunid crabs, which would oppose the salt effect on the oxygen transport system and, possibly, maintain a respiratory balance. Unless other respiratory or environmental parameters change, the role of hemocyanin in aerobic respiration must be smaller at lower salinity.

TABLE II

Respiratory parameters of Limulus polyphemus *in waters of different salinity, calculated from data in Figure 1, Table I and Sullivan* et al. (*1974*).

	Salinity (‰)		
	32–33	15.4–16.5	8.8–9.5
1. Postbranchial oxyhemocyanin (%)			
high O_2	100	99	99
2. Postbranchial oxygen concentration (ml/100 ml)		3.49	
high O_2	2.95	3.83	3.15
reduced O_2	2.90		3.13
3. Prebranchial oxyhemocyanin (%)		69	
high O_2	72	58	81
reduced O_2	50		55
4. Prebranchial oxygen concentration (ml/100 ml)		2.30	
high O_2	2.00	2.13	2.42
reduced O_2	1.38		1.64
5. Postbranchial—prebranchial oxygen concentration (m/100 ml)		1.19	
high O_2	0.95	1.70	0.73
reduced O_2	1.52		1.49
6. Oxygen uptake (ml/kg-min)		0.991	
high O_2	1.00	0.767	1.215
7. Cardiac output (ml/kg-min)		83	
high O_2	105	45	166
reduced O_2	66		82
8. % oxygen uptake by hemocyanin		80	
high O_2	72	89	74
reduced O_2	89		89

Effect of acclimation salinity on oxygen transport

The data in Table I do not unequivocally demonstrate a change in hemocyanin concentration with acclimation salinity, although the interpretation is complicated by the body size bias of the samples. Animals weighing 1 kg or less have significantly lower amounts of hemocyanin in their blood ($P < 0.05$; Student's *t* test), regardless of acclimation salinity. Comparison of the data for animals of the same size acclimated to different salinities suggest an increase in hemocyanin concentration in dilute waters, but the data are too few for quantitative analysis. In the discussion below it is assumed that an increase in hemocyanin concentration, if it occurs, involves a proportional increase in chloride sensitive as well as chloride insensitive components (Sullivan *et al.*, 1974).

The values for hemocyanin concentration predict an oxygen carrying capacity [2.96 (± 0.28) ml/100 ml] which is higher than several of the values in the

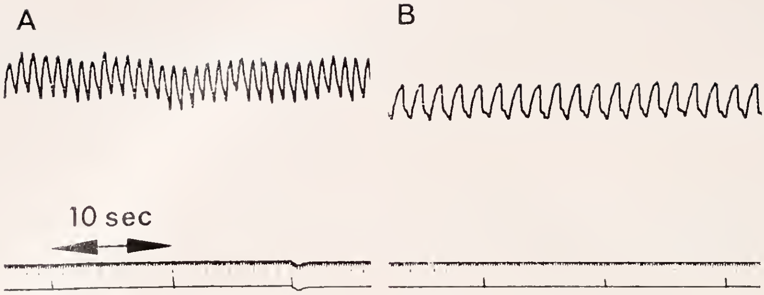

FIGURE 2. The effect of a reduction in salinity on heartbeat of *Limulus polyphemus*. (A) 32 (B) 22‰, four days later; 20–22° C; $PO_2 > 140$ torr.

literature (Redfield, Coolidge and Hurd, 1926; Mangum, Freadman and Johansen, 1975), but they are not significantly different from that mentioned by Sullivan *et al.* (1974). The animals studied by Mangum, Freadman and Johansen (1975) had been kept in the laboratory for at least three months, and it is very likely that the hemocyanin concentration was unnaturally low, due to starvation. Although the values in Table I are higher than those usually found in aquatic brachyurans, they are well within the range found in freshly caught or well fed shrimp (Djangmah, 1970) and in land crabs (Cameron and Mecklenberg, 1973).

Preliminary measurements of postbranchial blood PO_2 indicated that acclimation salinity does not appreciably affect the process of hemocyanin oxygenation at the gill, as long as ambient PO_2 is high (Table I). Subsequent observations were restricted to the parameters in prebranchial blood, which are more sensitive indices of changes in the per cent oxyhemocyanin.

The data for prebranchial blood PO_2 also reflect the body size bias of the samples (Table I). Larger animals clearly have lower prebranchial blood PO_2's, regardless of acclimation salinity ($P < .05$; Student's *t* test). Despite this confounding factor, the decrease in blood PO_2 with acclimation salinity is apparent; however, it is exaggerated in the data for 15.4–16.6 and minimized at 8.8–9.4‰. The response differs from that in blue crabs, which show a very small increase in blood PO_2 at low salinity (Weiland and Mangum, 1975).

The calculated parameters in Table II support the previous conclusion that, in well oxygenated waters ($PO_2 > 140$ torr), hemocyanin is fully oxygenated at the gill and it delivers only a small fraction of its oxygen to the tissues (Johansen and Petersen, 1975), despite the higher temperature and use of freshly collected animals in the present experiment. At an ambient PO_2 (98–105 torr) which is more typical of the bottom layer of water in the stream channels where *Limulus* enters the estuary (Deaton and Mangum, 1976), the role of hemocyanin becomes greater regardless of salinity, due to the changes in both pH and PO_2 (Johansen and Petersen, 1975; Table II).

The size bias of the samples becomes more important in interpreting the respiratory consequences of low salinity adaptation (Table II). If the results for 15.4–16.5 and 8.8–9.4‰ are combined, the mean body weight is about 2.2 kg, which is not significantly different from that of the high salinity sample, and the effects of low salinity on oxygen uptake and transport are not obscured. The lower blood PO_2 in dilute media induces greater deoxygenation of hemocyanin at the tissues (Table II). The predicted effect of low blood salt is in fact more than compensated, and the net result is an increase rather than a decrease in the volume of oxygen carried by hemocyanin. These calculations also suggest a decrease in blood flow at low salinity, which is consistent with recordings of heartbeat (Fig. 2). The measured decrease of heartbeat in different animals ranged from 13–33% ($P < .05$).

The respiratory consequences of the data in Tables I and II are summarized in Figure 3. Figure 3A shows the combined data for animals at low salinity, so that water PO_2 and body size are not variables. Figure 3B shows the data for animals of average size at $PO_2 > 140$ torr and 32‰ in comparison with those for small animals at $PO_2 = 98$–105 torr at 8.8–9.4‰, which more closely simulates the events occurring in natural estuarine migrations. Oxygen uptake is maintained at a nearly constant level, regardless of salinity and water PO_2, by decreases in blood flow and blood PO_2. Thus the mechanisms of respiratory adaptation at low salinity are in part passive, the increase in efficiency of oxygen transport by hemocyanin, and in part active, the bradycardia.

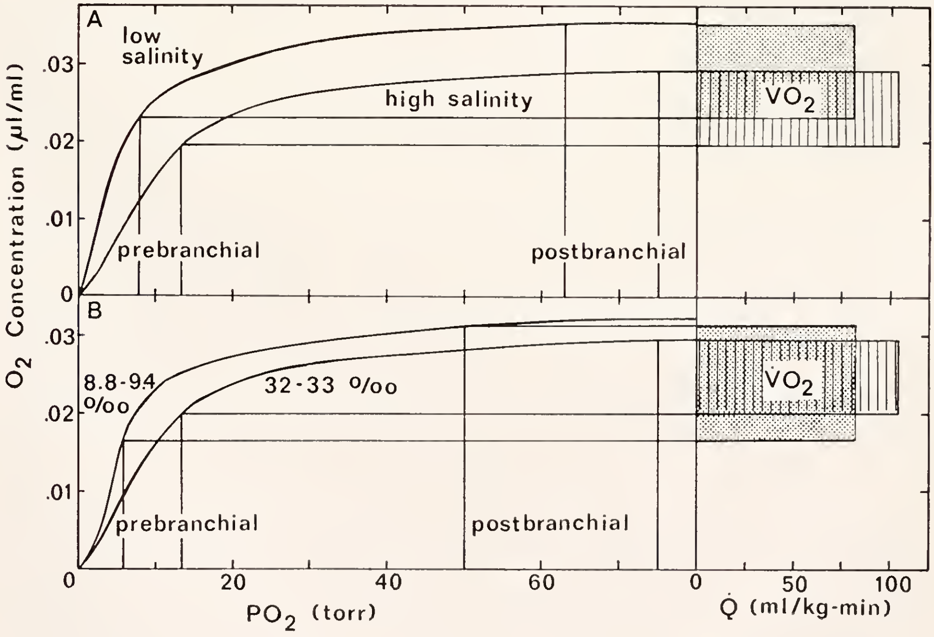

FIGURE 3. The changes in oxygen transport by the blood of *Limulus polyphemus* induced by adaptation to low salinity. The volume of oxygen delivered to tissue is represented by the height of the stippled or hatched boxes, and cardiac output ($\dot{Q}$) by the width. Thus, according to the Fick principle the area of the boxes represents oxygen consumption (µl/ kg-min). Curves are calculated from oxygen equilibrium data for *Limulus* hemocyanin (Redfield and Ingalls, 1933; Sullivan *et al.*, 1974), and from data in Tables I and II.

As indicated above, these conclusions rest upon the assumption that an increase in hemocyanin concentration at low salinity entails a proportional increase in each of the five electrophoretic components. If this assumption is incorrect, the error could have either of two effects. On the one hand, selective increase in the two chloride sensitive components would result in a greater difference between each member of the pairs of curves in Figure 3, and the increase in hemocyanin function might be mitigated or reversed. On the other hand, selective increase in the three chloride insensitive components, the more adaptive response, would result in less difference between the two curves and the response might be enhanced.

Discussion

The osmotic and ionic responses of the blood of *Limulus polyphemus* differ from those in decapod crustaceans only in quantitative terms. The animal osmotically conforms to a dilution of the medium at high salinities, and then begins to maintain a hyperosmotic blood at some point in the range 22–24‰ (Robertson, 1970). Unlike many crustaceans, however, the excretory organ contributes to the hyperosmotic condition of blood at very low salinity by forming a urine which is more dilute than blood. The coxal glands also contribute to acid-base regulation by forming a urine which is acidic to blood at 32–33 and 15.4–16.5‰, and alkaline at 8.8–9.4‰. Since the acid-base status of crustacean urine is not known, this comparison cannot be made.

The major difference in low salinity adaptation of *Limulus* and the portunid crabs seems to be the mechanism of osmotic adjustment. Salt absorption in *Limulus* cannot be attributed to a Na^+–NH_4^+ exchange, and the adjustment of intracellular fluids cannot be attributed in large part to the deamination of a pool of free amino acids.

The net result of the respiratory response to low salinity is a stability of aerobic metabolism, rather than the increase which occurs in portunid crabs (Siebers *et al.,* 1972; Mangum, 1976). In *Limulus* the effect of reduced salts on hemocyanin oxygenation is not opposed by a large increase in blood pH. The very small change may be due to the input of alkaline substances from the cells; it is unlikely to be explained by the protonation of more NH_3 because the ammonia concentrations of both blood and urine are reduced at low salinity. Instead, the effect of the reduction of blood salts on hemocyanin oxygenation is opposed by a decrease in blood PO_2, brought about by bradycardia. The response of ventilation to a change in acclimation salinity is not known, but it may also play a role in maintaining a stable rate of oxygen consumption. The locomotor movements appear to slow down in dilute waters and the book gill movements may do so as well.

The net respiratory balance in *Limulus* is not very different from that in portunid crabs. In both *Carcinus maenas* and *Callinectes sapidus,* oxygen consumption increases in the lowest part of their salinity range (which does not occur in *Limulus*), but the rate remains unchanged in *C. sapidus* over a very wide range of acclimation salinities (Siebers *et al.,* 1972; Laird and Haefner, 1976; Mangum, 1976.)

If blood PO_2 in *Limulus* did not change at low salinity, the function of hemocyanin would be greatly diminished, both in highly oxygenated waters and in the stream channels of estuaries. The salt effect on *Limulus* hemocyanin is so large that it would not be opposed completely by an elevation of pH, even one as great as that in *C. maenas* (0.3) or *C. sapidus* (0.2). In fact the large changes in salts and PO_2, and the small change in pH result in a greater importance of the oxygen carrying pigment when the animal enters dilute waters.

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SUMMARY

1. Oxygen affinity of *Limulus* hemocyanin is lowered by the addition of Cl^- and raised by the addition of H^+. Therefore the oxygen transport system should be influenced by ionic changes in the blood, such as those which accompany the spring migrations into estuaries.

2. When *Limulus* enters dilute waters, blood NaCl is reduced by almost half, even though active hyperosmotic regulation occurs. At very low salinities, the excretory organ plays a role in the imperfect regulation of blood NaCl by forming a dilute urine.

3. Unlike crustaceans, H^+ concentration of blood changes very little at low salinity, because little additional NH_3 is produced and no appreciable increase in H^+ binding to molecular NH_3 occurs. This response is believed to be related to the small role of free amino acids in intracellular osmotic adjustment.

4. Blood PO_2 decreases in low salinity as a result of bradycardia. While the oxygenation of hemocyanin at the gill does not change appreciably, deoxygenation at the tissues is enhanced and thus, in dilute waters, the pigment becomes more important in oxygen transport. This response, which also differs from that in crustaceans, occurs both in highly oxygenated waters and at the oxygen levels that are characteristic of the stream channels in the natural habitat.

5. The adaptation consists of concomitant changes which have opposite but counterbalancing effects on oxygen consumption in an unstable ionic environment.

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LUNAR AND TIDAL PERIODICITY OF MOLTING AND REPRODUCTION IN STOMATOPOD CRUSTACEA: A SELFISH HERD HYPOTHESIS

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One of the constraints imposed upon arthropods by their protective exoskeleton is the necessity to molt. While the physiology and morphogenesis associated with the molt cycle in crustaceans has commanded a great deal of attention (*e.g.,* Passano, 1960; Drach and Tchernigovtzeff, 1967; Stevenson, 1972; Aiken, 1973; Davis, Fyhn, and Fyhn, 1973; Reaka, 1975a), few workers have examined the process of molting in an evolutionary perspective. This study examines the ecological and evolutionary consequences of molting in stomatopod crustaceans.

Mantis shrimp are primitive members of the Malacostraca (Brooks, 1962; Siewing, 1963; Holthuis and Manning, 1969; Schram, 1969a, b, 1973; Burnett and Hessler, 1973; Reaka, 1975b), but they possess a highly specialized life style which may be associated with unique molting and reproductive adaptations. Stomatopods frequently live in species assemblages where as many as five congenerics occupy indistinguishable microhabitats in one tidepool (Reaka, 1972, and in preparation) and seven congenerics may occupy one tide flat (this study). Protection from predation is afforded by burrows or cavities in mud, sand, rocks or coral. Stomatopods employ intense and sometimes lethal agonistic behavior against conspecifics and congenerics while fighting to obtain or retain burrows and in addition are rapacious carnivores.

All crustaceans are susceptible to conspecific or other predators when molting and often possess behavioral adaptations to minimize mortality at this time (Passano, 1960). This vulnerability is considerably magnified in molting stomatopods because of their behavior and frequent sympatry with closely related species; if not killed by another mantis shrimp, a stomatopod may be displaced from its hole and exposed to predation by fishes (Townsley, 1953; Camp, 1973). Large size is an obvious advantage for pursuit and overpowering of prey, defense against predators, conspecific and congeneric fighting, and reproduction (Reaka, 1974, 1975c), but carries the disadvantage of repeated molts. How, then, do stomatopods minimize the effects of molting while maintaining their tactics of predation and contest competition? Reclusive and submissive behavior, as well as sealing the entrance of the burrow, reduce vulnerability at the molt in stomatopods; the stages of morphogenesis associated with incapacitation and exuviation are abbreviated in several aggressive taxa of crustaceans but especially in stomatopods; and asymmetrical stages of the molt cycle suggest that the early premolt may be a waiting period, whereupon initiation of the molt progresses rapidly following the correct cue (Reaka, 1975a). The present paper examines timing of molting and reproduction, and suggests that lunar and tidal synchrony of molting in the population may reduce mortality due to conspecific and congeneric aggression according to a temporal model of the selfish herd hypothesis (Hamilton, 1971).

Materials and Methods

Data on field and laboratory molting and reproduction were collected over several years in populations of *Gonodactylus zacae* (La Paz Bay, Gulf of California, Mexico). *G. zacae* was returned immediately to Berkeley where individuals were isolated in plastic aquaria at room temperature (19–26° C) with normal laboratory illumination. Group 1 ($N = 36$) was maintained from June 1971 until February 1972; group 2 ($N = 31$) from November 1971 until July 1972; group 3 ($N = 51$) from March 1972 until September 1972; group 4 ($N = 83$) from October 1972 to July 1973; group 5 ($N = 27$) from May 1973 to September 1973.

G. falcatus from Kaneohe Bay, Hawaii, was maintained in the laboratory at the Hawaii Institute of Marine Biology (HIMB) in similar conditions (about 26° C, laboratory illumination). In addition, some individuals occupied perforated containers in circulating seawater tables (about 26° C, natural photoperiod). *G. falcatus* was maintained in Hawaii during August, September, and early October (1971), September (1972), and October (1973), before shipment to Berkeley where maintenance was as described for *G. zacae* (group 1, $N = 149$, was maintained until August 1972; group 2, $N = 59$, until April 1973; group 3, $N = 54$, until March 1974).

Haptosquilla glyptocercus from Eniwetok Atoll, Marshall Islands, was maintained in closed containers at the Eniwetok Marine Biological Laboratory (about 27° C, natural photoperiod) during August and September 1972 before being shipped to Berkeley and maintained as described above until April 1973.

Pseudosquilla ciliata from Kaneohe Bay, Hawaii, was maintained at HIMB during September and early October 1971, and at Berkeley until March 1972 ($N = 11$); and also at Berkeley from February until June 1974 ($N = 38$). The conditions in which *P. ciliata*, shipped from Panacea, Florida, was maintained in Berkeley (February to June 1974, $N = 32$) were the same as described above.

Field and laboratory data from one Australian gonodactylid community include five species which were collected from Cockle Bay and Geoffrey Bay on Magnetic Island near Townsville, Queensland, from August until October 1973. In Australia these species were maintained in the laboratory (about 23° C, natural photoperiod and sometimes artificial lighting) in either plastic aquaria, or briefly in perforated plastic bottles floated in circulating sea water aquaria. After shipment to Berkeley, they were maintained as described above until April 1974.

Data on molting and reproduction from a similar community of gonodactylids in Thailand includes seven species from one reef flat. These species were collected and maintained in the laboratory at the Phuket Marine Biological Center from July until August 1973, and from June until August 1974, using the same procedures described above (normal laboratory illumination and laboratory water temperatures approximating 26° C).

The size range among the ten species of gonodactylids presented in this study varies from juveniles (10 mm total length) to adults (90–100 mm total length in the largest species). Molting rates do not vary over ontogeny within species of stomatopods; therefore the size distribution of individuals maintained did not

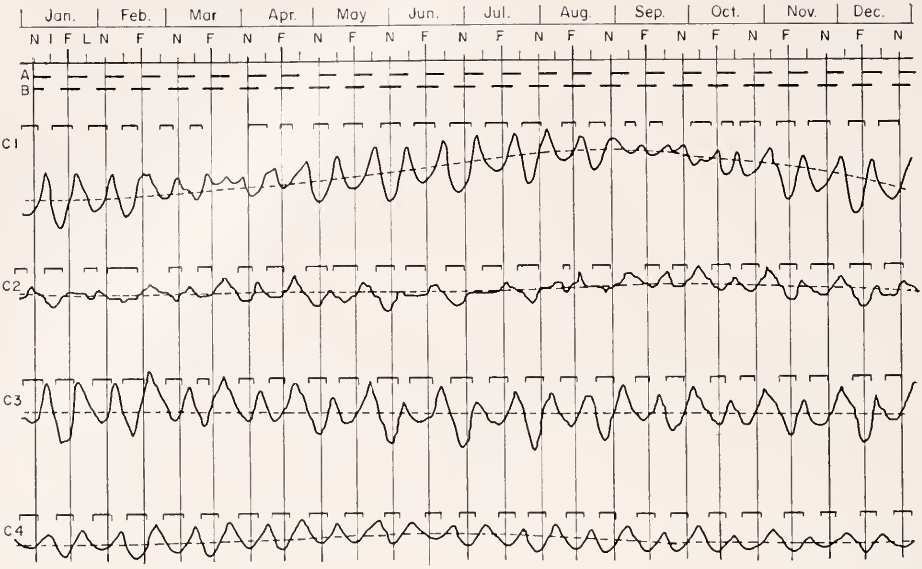

FIGURE 1. Lunar and tidal cycles at various sites for 1973, showing time divisions of the initial lunar (A), the divided lunar (B), and actual tidal (C) methods of analysis. C1 represents tidal curve at Guaymas, Mexico; C2, Honolulu, Hawaii; C3, Townsville, Australia; and C4, Phuket, Thailand. Dashed lines represent mean tidal levels used to define spring and neap tides. Differences in extremity of tidal curves are due to differences in the base tidal datum used in different countries.

affect incidence of molting. All animals were maintained individually in containers of appropriate size and volume (10 × 10 × 10 cm and 10 × 10 × 20 cm plastic boxes, and plastic bowls and aquaria of various sizes). All containers were provided with sand or gravel substrate and an Erlenmeyer flask painted black or pieces of coral for habitation. Individuals were fed pieces of fish, crustaceans, and mollusks twice or thrice weekly, and the sea water was changed later the same day. In all cases maintenance temperatures approximated field conditions. Although temperature and photoperiod were not rigorously controlled, they should have produced no systematic bias across lunar or tidal phases. Individuals were checked for molts and eggs daily or at most every two days.

When dealing with a temporally variable phenomenon, the problem arises of how to construct categories which encompass most of the events clustered in time rather than categories which bisect and therefore disguise the frequency modalities. This study used three methods of analysis which divided the lunar and tidal month into equal, logically derived units, and which were devised before beginning data analysis. Therefore, use of one or the other method does not represent any transformation or exaggeration of categories but indicates different ways of dividing a month into four equal divisions in order to encompass existing modalities. Figure 1 demonstrates the "initial lunar method," the "divided lunar method," and the "actual tidal method" of analysis. For the initial lunar analysis, a lunar calendar was used to identify the apex of the new (N), first (I), full (F), and last (L) quarter of each lunar month. This day was defined as the initial day of that lunar phase, and all molts or eggs were recorded which fell on that day and in the subsequent six to seven days before the apex of the next phase. This method therefore included events which occurred during the transitional stages between lunar phases, which may serve as cues for organisms. The divided lunar method attempted to encompass events which occurred during a complete lunar phase; for example, molts which occurred in the three days prior to the apex of the full moon, on the day of the full moon, and during the four days subsequent to the full moon were included in the F category. NF and IL indicate that data are derived from N + F and I + L categories. For the actual tidal method of analysis, the lowest tide of each day was recorded from tide tables. A mean tidal curve for the year was drawn through the bimonthly tidal cycles thus derived (dashed line, Fig. 1). All days in which the daily low tide fell below this mean curve were defined as spring tides, and all days in which the daily low was greater than the mean curve were defined as neap tides. The mean tidal curve provided that approximately equal numbers of days were represented in the spring and neap tidal categories over the year, and incorporated seasonal variation in tidal heights in some areas, particularly the Gulf of California.

In the tables and text, an asterisk indicates $P < 0.05$ and a double asterisk indicates $P < 0.01$ that the null hypothesis is correct. All χ^2 tests were repeated using Yates correction for continuity (Siegel, 1956, p. 109). However, in no case did this calculation alter the level of statistical significance; also, Lewontin and Felsenstein (1965) indicate that this correction need not be used. Therefore, only the original χ^2 values are presented.

RESULTS

Repeated observations from field collecting and laboratory maintenance suggested a clustering in time of molts and batches of eggs laid. Table I summarizes molts and eggs laid during lunar and tidal phases over several years using the initial lunar method of analysis. It was impractical to construct and analyze exact tidal curves from all sites over several years. Therefore, in this table the tidal categories are the sum of the molts and eggs occurring during the two associated lunar phases. In the Gulf of California and Hawaii, NF phases (new and full, initial lunar method) correspond in general to neap tides or to the transition from spring to neap tidal cycles ("neap"), while IL (first and last) phases correlate with spring tides or to the transition from neap to spring tidal cycles ("spring"; see Fig. 1).

Gonodactylus zacae (*Gulf of California*)

In female *G. zacae* there is a strong bimodal trend in numbers of molts over the lunar cycle; most molts occur during "neap" tides (Table I). Males seem to show a monthly lunar rather than a tidal cycle, with reduced molting during the first quarter and increased molting around the last quarter ($L > I$, $\chi^2 = 5.00$*, $df = 1$). However, this may be an artifact of small sample size, since the frequency distributions of molting males and females may not be significantly different ($P < 0.10$) across lunar phases ($\chi^2 = 6.84$, $df = 3$) and tidal cycles ($\chi^2 = 3.75$, df = 1). Fewer molting males than females results from fewer males than females in the population rather than lower molting rates of males.

Although oviposition in *G. zacae* appears to occur more commonly during "neap" tides, these data fall below the 0.05 confidence level. However, the distribution of oviposition across tidal categories does not differ from that of molting in *G. zacae,* either in females ($\chi^2 = 0.23$, df = 1) or in the total population ($\chi^2 = 0.00$, df = 1). Also, the distribution of oviposition across "spring" and "neap" tidal phases in *G. zacae* is not significantly different from oviposition patterns in *G. falcatus* ($\chi^2 = 0.35$, df = 1).

Each of the five groups of *G. zacae* was collected during a single lunar phase or a transitional phase. In group 1 (collected during L) a total of 13, 5, 11, 8 molts occurred in the four respective lunar phases; for groups 2 and 4 (collected during I), a total of 27, 10, 24, 11 molts occurred in the four lunar phases; and 4, 1, 7, 8 and 3, 3, 5, 2 molts occurred in groups 3 and 5 which were collected during the I–F transition and during N, respectively. Therefore, time of collection does not appear to set the molting rhythm. There is no apparent change in the pattern of molting in lunar periods with increased time spent in laboratory conditions. Molting rates do not decline with increased maintenance time; and there is no demonstrable effect of time of year or reproductive season upon number of molts or intermolt interval (Reaka, 1975c, and in preparation).

Stomatopods do not cement eggs to appendages and have no obligatory molt before oviposition. In three cases in which female *G. zacae* laid eggs between molts in the laboratory, oviposition occurred 41%, 51%, and 59% through the intermolt intervals of several months; and one *G. zacae* was found in the field with eggs at the eyespot stage (probably one to two weeks old) and with an exuvium in her cavity. Therefore, it seems unlikely that there is a relationship between oviposition and a stage of the molt cycle which might explain the molting synchrony of females. Eggs from *G. zacae* were found in the field or in the laboratory only from March 31 through November, although animals were maintained year round. There is no significant difference ($\chi^2 = 3.59$, df = 3) between lunar molting synchrony of females during the eight months of this reproductive season (22, 9, 27, 12, respectively) and the nonreproductive period (15, 5, 7, 4, respectively).

In summary, the bimodal molting synchrony of female *G. zacae* does not appear to be due to collecting artifact, laboratory conditions, reproductive patterns or seasons. Males of *G. zacae* either show a monthly molting pattern or the smaller number of molting males is insufficient to demonstrate the bimodal pattern observed in the more abundant females. Among both males and females, 65% of all molts occur during N and F lunar phases associated with "neap" tides in the Gulf of California. Further observations in the field are required to verify whether or not oviposition is correlated with lunar or tidal cycles. The divided lunar method of analysis produces only slightly less extreme patterns in the temporal distribution of molts and eggs in *G. zacae.*

G. falcatus (*Hawaii*)

In *G. falcatus* from Hawaii (Table I) no lunar or tidal molting synchrony can be demonstrated statistically, although there is a trend for both males and

TABLE 1

Molting and reproductive patterns in species of stomatopods. N, I, F, L indicate the new, first, full, and last phases of the lunar cycle; SPR, N indicate spring and neap tidal cycles. The initial lunar method of analysis was used for these calculations. Critical values for $P < 0.05$ *for the* X^2 *test are 3.84* ($df = 1$) *and 7.82* ($df = 3$).

		Molts								Eggs							
		N	I	F	L	X^2	NP	SPR	X^2	N	I	F	L	X^2	NP	SPR	X^2
G. zacae	female	37	14	34	16	16.90**	71	30	16.64**	6	5	11	4	4.46	17	9	2.46
	male	10	5	13	15	5.27	23	20	0.20								
	total	47	19	47	31	15.44**	94	50	13.44**								
G. falcatus	female	23	34	28	30	2.18	51	64	1.46	5	5	9	0	—	14	5	4.26*
	male	17	23	11	16	4.34	28	39	1.80								
	total	40	57	39	46	4.50	79	103	3.16								
H. glyptocercus	total	5	2	5	1	—	10	3	3.76								
P. ciliata																	
Hawaii	female	3	4	1	7	—	4	11	3.26	1	0	1	0	—	2	0	—
	male	4	5	3	10	—	7	15	2.0								
	total	7	9	4	17	9.25*	11	26	6.08*								
Florida	female	1	0	1	6	—	2	6	—	2	0	5	0	—	7	0	—
	male	1	1	0	1	—	1	2	—								
	total	2	1	1	7	—	3	8	2.27								
Total		9	10	5	24	17.16**	14	34	8.33**	3	0	6	0	—	9	0	—

females to molt during "spring" (57%) rather than "neap" (43%) tidal cycles. The distribution of molts across tidal cycles differs significantly from that in *G. zacae* ($\chi^2 = 15.44^{**}$, df = 1) and *H. glyptocercus* ($\chi^2 = 5.49^{*}$, df = 1), but not from *P. ciliata* ($\chi^2 = 3.19$, df = 1), substantiating the tendency for *G. falcatus* to molt during "spring" tides.

Significantly more female *G. falcatus* lay eggs during "neap" than "spring" tides. Egg deposition occurs in significantly different tidal phases from molts in females ($\chi^2 = 5.62^{*}$, df = 1) and from molts in the population ($\chi^2 = 6.34^{*}$, df = 1).

Again, these data span different seasons of different years. Reproduction occurs year round in *G. falcatus* (personal observation; Kinzie, 1968). The failure to demonstrate significant synchrony of molting does not appear to be due to fading of the pattern, either at particular times of the year or after laboratory maintenance. All data also were analyzed using the divided lunar method, but no significant change in clustering of events in time was observed.

Haptosquilla glyptocercus (*Eniwetok*)

H. glyptocercus demonstrates a strong tendency to molt during "neap" tides (Table I), although the data fall barely below the 0.05 level of significance. The distribution of molting in tidal cycles does not differ from that in *G. zacae* ($\chi^2 = 0.72$, df = 1) but diverges from the tidal molting patterns observed in *G. falcatus* ($\chi^2 = 5.49^{*}$, df = 1) and *P. ciliata* ($\chi^2 = 9.77^{**}$, df = 1).

Pseudosquilla ciliata (*Hawaii, Florida, Thailand*)

P. ciliata, a circumtropical gonodactylid which is sympatric with *G. falcatus* in Hawaii, molts significantly more often during "spring" than "neap" tidal cycles (Table I). Populations of *P. ciliata* from Hawaii and Florida show similar patterns of molt timing ($\chi^2 = 1.50$, df = 3); most molts occur during the last phase of the moon. Eggs are laid during "neap" tides. A yearly and seasonal breakdown of these data, as well as lunar phases at collection, shows no relationship between collection and molting patterns.

During maintenance of *P. ciliata* in Thailand 2, 1, 1, 5 individuals molted in the N, I, F, L lunar periods, respectively (initial lunar method; note that Table III presents only field data). This pattern is not significantly different from that of *P. ciliata* from Hawaii and Florida ($\chi^2 = 0.45$, df = 3). However, in Thailand seven batches of eggs were laid in the laboratory during L (neap-spring transition during June–August), and one batch was deposited in F.

Species from Australia

Table II shows molting and reproduction in five sympatric species of Australian gonodactylids. The distribution of molting during spring and neap tidal periods does not differ among the five species ($\chi^2 = 4.71$, df = 4), although the sibling species *G. graphurus* and *G. falcatus* probably show different molting patterns ($\chi^2 = 3.65$, df = 1; $P = 0.04$, Fisher exact probability test). No lunar or tidal molting synchrony of the stomatopod community is demonstrated. Molting sample sizes are small, and the transfer during maintenance from the southern to the northern hemisphere could obscure a molting pattern if present. Molting was also

TABLE II

Molting and reproduction in five species of Gonodactylus *from Australia. Analysis is by initial lunar and actual tidal cycle methods.*

	Molts								Eggs							
	Initial lunar					Actual tidal			Initial lunar					Actual tidal		
	N	I	F	L	X^2	SPR	NP	X^2	N	I	F	L	X^2	SPR	NP	X^2
G. graphurus	7	7	6	2	3.09	7	15	3.10	0	4	0	4	—	7	1	—
G. falcatus	4	2	4	4	—	9	5	1.14	1	2	1	1	—	2	3	—
G. smithii	1	1	0	2	—	2	2	—	1	0	1	0	—	2	0	—
G. hendersoni	3	0	1	0	—	1	3	—	0	0	0	1	—	1	0	—
G. affinis	2	2	0	3	—	4	3	—	0	2	1	0	—	3	0	—
Total	17	12	11	11	1.94	23	28	0.49	2	8	3	6	4.78	15	4	6.36*

analyzed using the divided lunar method, and no change in clumping of events was observed. *G. graphurus* molts and lays eggs in opposite tidal cycles ($P = 0.009$, Fisher exact probability). Although untestable, time of egg deposition does not appear to vary among species. Oviposition across tidal phases is not significantly different in *G. graphurus* and *G. falcatus* from Australia ($P = 0.11$, Fisher exact probability). Analysis of egg deposition by all species across all four lunar categories, using both initial and divided lunar methods, fails to achieve statistical significance. Using the initial lunar method, IL > NF ($\chi^2 = 5.56^*$, df = 1), indicating a probable correlation of egg laying with spring tides or the transition from neap to spring tides (see Fig. 1). Using actual tidal data, eggs of the five species occur significantly more frequently during spring than neap tidal cycles (Table II). All eggs except for one batch from *G. graphurus* were deposited in the field and laboratory in Australia soon after collection rather than after transport to North America.

Species from Thailand

Seven species of sympatric gonodactylids were collected and maintained in Phuket, Thailand. In contrast to the conditions for all of the above species, the period of maintenance was short. Large numbers of animals were differentially collected, maintained, and sacrificed during lunar cycles of these periods; therefore, the numbers alive during each lunar period were not necessarily comparable. To avoid this bias, Table III includes only field data. The number of individuals collected during each lunar or tidal phase provide the proportion of the molts and egg batches expected to occur in field collections during that phase for each species. In 1973, 77.8% of all individuals (N = 447) of all species were collected during new and full phases of the moon, but 100% of all molts (N = 8) found in the field occurred during new and full phases of the moon (initial lunar method). Molting showed similar patterns in 1973 and 1974. Table IIIA compares observed and expected frequencies of molting (1973 and 1974) during the transition from spring to neap tides (NF) and from neap to spring tides (IL), using the initial lunar method of analysis (Fig. 1). Data were analyzed also using divided lunar and actual tidal methods.

Field data alone demonstrate synchronization of molting with waning spring tides in *G. chiragra* (the only species for which sufficient numbers of molts allow testing). Analysis by the actual tidal method also shows significant correlation of molting in *G. chiragra* with spring tides ($\chi^2 = 10.72^{**}$, df = 1).

Also, significantly more individuals than expected in the Thailand stomatopod community molt during the transition from spring to neap tides (NF, initial lunar) and during spring tides (actual tidal method, $\chi^2 = 7.76^{**}$, df = 1). Although untestable, no significant differences in molt timing are apparent among species of the community, except perhaps *H. glyptocercus* and *P. ciliata*. Even if these two species do not follow the tendency for the rest of the community to molt during the transition from spring to neap tides or during spring tides, their influence is to bias the sample toward the null hypothesis rather than the alternate hypothesis.

If molts which occur within one to two weeks of capture are included in these field data, significantly more molts than expected still occur during spring than

TABLE III

Molting and reproduction in seven species of Gonodactylidae from Thailand. Analysis is by the initial lunar method for molting, and by the divided lunar method for oviposition. Only molts and eggs found in the field are included. The number of individuals collected during each tidal phase predicts the number of molts and eggs expected during that phase.

A. *Molts observed and expected in the field* (**1973** + **1974**).

	Number Collected		NF		1L		χ^2
	NF	1L	Observed	Expected	Observed	Expected	
G. viridis	232	181	6	(3.9)	1	(3.1)	—
G. chiragra	143	199	18	(9.6)	5	(13.4)	12.61**
G. smithii	99	7	1	(0.9)	0	(0.1)	—
G. falcatus	101	11	3	(2.7)	0	(0.3)	—
G. ternatensis	34	25	5	(4.6)	3	(3.4)	—
H. glyptocercus	56	37	0	(1.2)	2	(0.8)	—
P. ciliata	66	21	0	(0.8)	1	(0.2)	—
Total	731	481	33	(23.7)	12	(21.3)	7.71**
B. *Fresh egg batches observed and expected in the field.*							
(**1973**)							
G. viridis	161	30	10	(8.4)	0	(1.6)	—
G. chiragra	75	38	1	(0.7)	1	(0.3)	—
G. smithii	44	1	9	(8.8)	0	(0.2)	—
G. falcatus	44	10	5	(4.1)	0	(0.9)	—
H. glyptocercus	25	19	1	(0.6)	0	(0.4)	—
Total	349	98	26	(21.1)	1	(5.9)	5.29**
(**1974**)							
G. viridis	208	14	16	(15.9)	1	(0.1)	—
G. chiragra	159	70	0	(2.1)	3	(0.9)	—
G. smithii	57	4	2	(1.9)	0	(0.1)	—
G. falcatus	46	12	4	(3.2)	0	(0.8)	—
G. ternatensis	44	15	8	(6.0)	0	(2.0)	—
H. glyptocerus	42	7	7	(6.0)	0	(1.0)	—
P. ciliata	65	7	1	(0.9)	0	(0.1)	—
Total	621	129	38	(36.0)	4	(5.0)	0.31

neap tides in the community (actual tidal method, $\chi^2 = 4.78^*$, df = 1; N = 57 molts). However, laboratory maintenance increases scatter across lunar and tidal phases. Therefore, the effect of laboratory maintenance in general (such as for *G. zacae, G. falcatus, P. ciliata,* and the Australian community) should have been conservative, favoring the null hypothesis of no molting synchrony.

Although 78.0% of the individuals were collected during NF (divided lunar method) in 1973, 96.3% of all egg batches found in the field occurred during new and full lunar phases associated with spring tides (see Fig. 1, Table III). Also, if laboratory data (with expected values calculated from the number of individuals of each species alive in the laboratory during a given lunar phase) are included, more egg batches than expected were laid during spring than neap tidal phases in

1973 ($\chi^2 = 22.78^{**}$, df = 1; N = 33 batches of eggs). Use of the initial lunar and actual tidal methods show similar but less extreme trends for both field and laboratory egg data. Similar analyses in 1974, however, fail to show significant lunar synchrony of oviposition (Table IIIB). The heavy collecting bias in favor of spring tides relative to the small number of egg batches may account for this discrepancy, but conclusions await further verification.

The validity of the hypothesis of molting and reproductive synchrony in all of the above populations may be refuted by demonstrating bias in collecting and maintenance. Because of isolated maintenance conditions, social facilitation or chemical communication did not contribute to the results recorded in this study. There is no relationship between date of collection and pattern of molting during laboratory maintenance in any of the populations. Groups were collected during a single lunar phase, and it is difficult to see how this could impose a bimodal pattern of molting in the laboratory. Within species, groups collected in different lunar phases show similar molting patterns. Also specimens of *G. zacae, G. falcatus*, and *P. ciliata* were maintained in the laboratory under the same conditions at the same time. While *G. zacae* shows a strong bimodal molting pattern associated with neap tides, *G. falcatus* and *P. ciliata* exhibit a significantly different molting periodicity, indicating that laboratory regimes do not impose the observed molting pattern. In addition, field data from Thailand demonstrate tidal molting synchrony in *G. chiragra* and in the stomatopod community as a whole, excluding the effect of maintenance conditions. In fact, inclusion of molting data from the laboratory increases variation in the temporal pattern of molting. Hence, data analyzed from laboratory maintenance probably represent a conservative estimate of molting synchrony in the population.

The molting pattern does not seem to be biased by tidal reproductive rhythms, since oviposition does not appear to be correlated with a physiological stage of the molt cycle, and in *G. zacae* the molting rhythm does not differ in reproductive and nonreproductive seasons. Different species show no consistent relationship between the lunar-tidal phase of molting and reproduction: *G. zacae* molts and probably reproduces during "neap" tides. During maintenance in Thailand *P. ciliata* appears to both molt and oviposit during neap-spring transitional tides or last lunar phases. *P. ciliata* from Hawaii and Florida molts during "spring" tides (or the last lunar phase) but lays eggs during "neap" tidal cycles, and *G. falcatus* shows the same tendencies. *G. graphurus* molts during neap but oviposits in spring tidal cycles. In addition, molting and egg laying probably occur too infrequently to be intimately related, although they may be independently mediated by similar environmental cues. Molts occur about every three months to once annually in *G. zacae* and *G. falcatus,* and may occur only every two years or more in smaller species. This author has never observed any female stomatopod to lay more than one batch of eggs in a year in the laboratory, although about 100 individuals of *G. zacae* and *G. falcatus* were maintained in the laboratory for a year or longer. Agonistic behavior and coloration of the ovaries indicate that field populations of gonodactylids always have a high percentage of nonreproductive females even at the peak of the long or continuous reproductive season; the percentages of females with egg batches found in the field suggest that females of most species reproduce about once per year or less (Reaka, 1975c), although the population may reproduce in biannual peaks (Kinzie, 1968). Therefore, reproduction cannot be shown to structure tidal molting patterns.

In spite of the above arguments, the molting data derived from extensive periods in the laboratory may not be independent in some way which would make statistical testing based upon subsequent lunar or tidal groupings untenable (J. T. Enright, Scripps Institute of Oceanography, personal communication). There are several arguments against this objection to the data. First, I can identify no cause for lack of independence among molts which occur in the laboratory. Second, if a spurious correlation of molting data with a tidal cycle results from the influence of pooling data or from some unidentified extraneous factor, then one might expect this lack of independence to affect the grouped results in one species by chance, but it should not recur among the several different species which were maintained at different times and different years in the laboratory (Table I). Third, and most important, the results of molting in the laboratory are consistent with field data (Table III), and with tidal synchrony of reproduction. Oviposition series usually occurred within a relatively short period and therefore were not subject to factors acting over longer intervals which might introduce dependence among events and artificial grouping. Also, the laboratory data are intended to provide more complete information on the general problem of molting and reproductive synchrony in stomatopod populations, and not to argue that endogenous molting and reproductive rhythms persist precisely in phase with the moon for extensive periods in constant conditions. Detailed records of molting and reproduction are available in Reaka (1975c) or upon request from the author.

In conclusion, one is left to reject the null hypothesis of no molting synchrony until further verification.

DISCUSSION

A great body of literature supports the existence of biological clocks which govern a variety of cyclic functions in protists, plants and animals. Evidence supports both endogenous and exogenous control of these rhythms (*e.g.*, Brown, Hastings, and Palmer, 1970; Enright, 1970; Brown, 1972; Palmer, 1973, 1974). Most studies of tidal rhythms have concentrated on daily lunar or tidal cycles ("circalunadian" rhythms; Palmer, 1973). The significance of studies of circalunadian rhythms to the semi-lunar and tidal rhythms of molting and reproduction in stomatopods is first, that circalunadian rhythms, like circadian rhythms, have been shown to persist for remarkably long spans of time relative to their period length in controlled conditions, indicating either a strong endogenous component, an enduring entrainment effect from lunar or tidal conditions prior to or at collection, or a continuing environmental stimulus transcending "constant conditions" (see Reaka, 1975c, for a detailed discussion of this literature). Therefore, it seems reasonable to hypothesize that lunar or tidal rhythms occur in stomatopods, and that the length of time these rhythms persist (even weakly) in the laboratory, relative to the phase length of the environmental periodicity, is not unusual compared to the circalunadian rhythms exhibited by many marine species.

Secondly, the extensive literature on circalunadian rhythms provides information about proximate environmental factors which may act upon lunar and tidal rhythms (see Reaka, 1975c, for discussion). Inundation and exposure have little effect in general upon entrainment of circalunadian rhythms; it seems unlikely that exposure during collection induced artificial molting and reproductive rhythms. Photoperiod and maintenance temperature in general have little effect upon circalunadian rhythms. Extreme temperature pulses or regular temperature cycles are known to induce circalunadian rhythms, but did not occur during collection and maintenance of mantis shrimp. Pressure changes and mechanical agitation may entrain circalunadian rhythms, are associated with changing tides, and could occur in the natural environment prior to collection of the species in this study; however, these should not have introduced artifacts during maintenance in the laboratory. It seems probable that local tidal conditions, perhaps in conjunction with innate cyclic processes (and pervading lunar effects cannot be ruled out), entrain molting and egg laying in the shallow reef habitat and that these physiological processes persist in the laboratory unaffected by collection.

The factors controlling longer term rhythms, such as semi-lunar, lunar, tidal, seasonal, or annual cycles, are more difficult to identify than those for daily rhythms. Tidal rhythms offer several difficulties since tides may be unpredictable, either daily or monthly, due to local topographical features as well as geographical area. Storms are common in tropical seas and have major effects upon local tides as well as upon photoperiods resulting from moonlight (see Barnwell, 1976, for discussion of the complexities of tidal form and implications for concepts of rhythms.) There is probably a selective advantage for a marine animal to possess a rather strong endogenous component, at least persistent entrainment to long term lunar or tidal cyclic phenomena. A scattered literature on long term lunar and tidal rhythms of activity, reproduction, and molting provides important information for evaluating the proximate factors which may affect rhythms in stomatopods and the ultimate factors which may operate in selection for reproductive and molting rhythms.

A sand beach isopod, *Excirolana chiltoni,* shows persistent daily and monthly activity rhythms which are probably entrained by tidal height and mechanical agitation in association with an endogenous rhythm; light probably does not control these rhythms (Enright, 1972). A shrimp, *Anchistioides antiguensis,* swarms at the surface on nights following the new moon (Wheeler, 1937).

The correlation of reproduction with lunar cycles in marine species has been noted in a number of reviews (Korringa, 1947, 1957; Enright, 1970; Giese and Pearse, 1974). The European oyster releases larvae semi-monthly (Korringa, 1947, 1957). Synchronous courting and mating around the new and full moon in fiddler crabs (*Uca annulipes, U. triangularis*) allows the release of planktonic larvae on spring tides (von Hagen, 1970). A tropical, tree-dwelling grapsid crab (*Aratus pisoni*) and a land crab (*Cardisoma guanhumi*) release larvae during new and full moon periods (Warner, 1967; Gifford, 1962). The grunion (*Leurestes tenuis*) spawns on sand beaches in California one to three hours after high tide a few days after each new moon from February to September (Walker, 1952). Protected from predation, the larvae develop in wet sand until they emerge during the next spring tidal cycle. At least two other species of fish (*Hubbsiella sardina, Galaxia attenuatus*) show lunar spawning (Walker, 1952).

Reproductive Pacific and Atlantic palolo worms of several genera swarm during specific lunar cycles of several months a year. Swarming in the Atlantic palolo, *Palola* (*Leodice*) *fucata,* occurs a few hours after sunset of the first or more commonly the last quarter of the moon (Clark and Hess, 1940). *Platynereis dumerilii,* a polychaete from the Mediterranean, normally spawns during the new moon (Hauenschild, 1960). These studies suggest that maturity of the worm, water turbulence, and changes in photoperiod associated with moonlight control the lunar synchrony of spawning which results in synchronous development and subsequent reproduction. These rhythms have strong endogenous components and continue for a number of months in the absence of external cues, particularly if imprinting of the rhythm occurs early in the life of the individual.

Monthly or semi-monthly reproductive rhythms occur in some species of echinoderms but appear to be absent in other species. Populations of a sea urchin, *Diadema setosum,* from different areas of the Indo-Pacific show either monthly spawning in different lunar phases (Fox, 1923; Yoshida, 1952; Pearse, 1968, 1975), no detectable reproductive synchrony (Stephenson, 1934; Mortensen, 1937; Pearse, 1969), or semi-monthly spawning (Kobayashi and Nakamura, 1967). Populations of another diadematid urchin, *Centrostephanus coronatus,* from the east Pacific reproduce in monthly reproductive synchrony, but spawn in different lunar phases in different years (Pearse, 1972, 1975), or in the same lunar but different tidal phases from year to year (Kennedy and Pearse, 1975).

An intertidal midge, *Clunio marinus,* pupates three to five days before maximum spring tides, emerges, mates and oviposits within a few hours of the extreme low tide during each new and full moon (Neumann, 1966, 1969). This genetically based reproductive rhythm is synchronized by tides in some populations and by photoperiod in others, and persists for several lunar cycles in constant conditions.

Several stages of the life cycle of a salt marsh pulmonate snail (*Melampus bidentatus*) are synchronized with spring high tides (Russell-Hunter, Apley, and Hunter, 1970, 1972). Individuals brought into the laboratory in the spring (lacking tidal submergence for ten semi-lunar cycles) aggregate, copulate, and lay eggs during the summer according to fortnightly cycles associated with the new and full moon. In the field larvae hatch in semi-lunar synchrony; hatching can be induced by several inundations. The larvae settle into the high intertidal habitat occupied by the adults two weeks later on high spring tides.

Excirolana chiltoni occurs in a narrow zone of sand on the high intertidal beach (Klapow, 1972). In addition to daily and fortnightly activity patterns, field studies of this isopod demonstrate semi-lunar rhythms of feeding, parasitic infection, mating, release of young, and molting. Feeding and parasitism cycles result from rather than cause the molting rhythm. Synchronous breeding results in synchronous release of young and synchronous molting of the first stage manca two weeks later. The reproductive periodicity apparently subserves the primary adaptation of molting synchrony. Molting in the developmental stages occurs in phase with the molting periodicity of the adult population, and molting synchrony does not diminish among older individuals, even those which are more than one year old. The molting peak occurs in the week prior to new and full moons on rising spring tides in field populations, and persists less distinctly through at least one molt cycle in the laboratory. During rising spring tides, molting isopods are exposed briefly to desiccation and predation; however, on maximum or receding spring tides molting individuals, stranded high on the beach and lacking the ability to burrow into the sand, probably are killed.

A rocky intertidal isopod, *Lygia oceanica*, molts during neap tidal phases (Glaçon, 1968). The rhythm persists in the laboratory for at least eight tidal cycles. I divided the data presented by Glaçon into seven-day periods which encompassed the spring or neap tides. In successive spring and neap tidal cycles 6, 59, 22, 56, 19, 44, 24, 45 molts occur, respectively ($\chi^2 = 75.12^{**}$, df = 7; and $\chi^2 = 64.32^{**}$, df = 1, for spring and neap categories). These data represent comparable percentages of molts (26% spring, 74% neap) to those observed in the tidal phases in this study. The rocky crevices inhabited by *L. oceanica* are submerged only a few days each month, when these semi-terrestrial isopods emigrate to higher terrain to escape wave action and prolonged submersion. Individuals which molt during the spring tidal phase probably perish.

Some evidence suggests that a molting rhythm occurs in a shrimp (*Anchistioides*) which swarms and mates on nights following the new moon (Wheeler, 1937; Klapow, 1972). Nouvel (1945) finds increased numbers of molts immediately after spring tides and during the approach of the first and last lunar phases in a shrimp (*Leander serratus*) maintained in the laboratory. The prevalence of reproductive periodicities, and the frequent obligate relationship between breeding and molting in crustaceans, suggests that molting rhythms may be more common than usually thought.

These studies provide several conclusions of significance for the present analysis of molting and reproduction in stomatopods. First, long term lunar and tidal rhythms in marine organisms persist in the laboratory in the absence of environmental cues and appear to have strong endogenous components. Secondly, long term rhythms of activity, reproduction and development, and molting sometimes are entrained by long photoperiods resulting from the full moon, by inundation from high tides, and by turbulence. These rhythms frequently are entrained early in life, and subsequent cycles result from this early synchronization. Thirdly, the reproductive rhythm appears to maintain the primary molting rhythm in sand beach isopods. Although young crustaceans sometimes molt faster than older individuals, the entire population of sand beach isopods remains in molting synchrony. Similar phenomena appear to occur in stomatopods. Fourthly, as in the present study, reproductive synchrony in different populations may occur in different lunar phases (*e.g.*, urchins). In different years one population may spawn during different lunar phases, or during the same lunar but different tidal phases. Different populations (as in *Clunio*) may be either genetically or environmentally entrained to use different cues; or a complex interaction of lunar, tidal, or other factors may result in molting or spawning during different lunar phases. The important selective factor for many species may be synchronous spawning or molting among individuals by social or whatever cues are available for a given population.

The primary selective advantage for long term lunar or tidal rhythms of activity probably rests in feeding efficiency and increased mating opportunity. Synchronous reproduction correlated with lunar or tidal cycles may provide maximum mating efficiency and fertilization of gametes, particularly among sedentary, cryptic, nonabundant species, or species with external fertilization; and reproductive synchrony may allow simultaneous development and molting. Tidal reproductive synchrony may be associated with exploiting an area of the intertidal otherwise unavailable where young or adults encounter low competition or predation (*e.g.*, grunion, midges). Availability of food for larvae and favorable currents for larval dispersal represent important but relatively unexamined factors which may govern tidal reproductive rhythms. Among intertidal organisms, physical factors such as temperature, dryness, and wave action may select for or against reproductive success of individuals which oviposit during spring or neap tidal cycles. Selection for synchronous molting in populations may be related to mating or may be imposed by physical damage, drowning, or desiccation when molts occur during inappropriate tidal cycles. Several hypotheses which may explain selection for molting and reproductive rhythms in stomatopods will now be examined.

If physical factors in intertidal or shallow water areas favor molting and reproductive rhythms, these rhythms should reflect the habitat and depth distributions of the respective species of stomatopods, and both molting and reproduction should occur during the same tidal cycle of least physical stress. Low intertidal regions suffer greatest exposure during spring tidal cycles but decreasing or no exposure during neap tidal cycles. Increasing height in the intertidal is accompanied by greater exposure during neap tidal cycles, whereas such areas are inundated once or twice daily during spring tides. Therefore, both molting and reproduction should occur during neap tides in species restricted to the low intertidal; in high intertidal species molting and reproduction should occur during spring tides.

Predation could impose selection for cyclicity in the shallow water environment, since time of exposure to fish predation is greatest during spring tides for high intertidal species, but greatest during neap tides for species occuring low in the intertidal. If displaced from their burrow, stomatopods would be most vulnerable to predators when incapacitated by brood care or especially by molting. Both molting and reproduction should occur in the opposite tidal cycle from the period of most intense predation. Therefore, species from low intertidal areas should molt and reproduce during spring tides, and species from high intertidal areas should molt and reproduce during neap tides.

Feeding cycles during tidal phases could impose selection for molting and reproductive rhythms. Feeding time for species which occur in the high intertidal area is restricted during spring tides, since individuals feed mostly on incoming and outgoing tides; during low water individuals remain in their burrows which are usually above the water line, and at high water fish predators abound in the area, making foraging dangerous. Feeding time for species in moderately high and in high intertidal areas is greatest during neap tides when shallow water excludes predators for a large percentage of the time. In low intertidal areas, feeding time is limited during spring tides by exposure at low water and by predators at high water, and throughout neap tides by predators. Stomatopods do not feed when molting or caring for eggs; molting and reproduction should be concentrated when feeding is disadvantageous or impossible. Species high in the intertidal should molt and reproduce during spring tides, and no synchrony is predicted for species from low intertidal areas.

Therefore, the *physical factors hypothesis* predicts molting and reproductive rhythms for high intertidal species during spring tides and for low intertidal species during neap tides; the *predation hypothesis* predicts rhythms for high intertidal species during neap tides and for low intertidal species during spring tides; and the *feeding time hypothesis* predicts rhythms for high intertidal species during spring tides, but no rhythms for low intertidal species. The following analysis of stomatopod distributions indicates confirmation (+) or contradiction (−) of the predictions from each of these hypotheses, respectively. For example (+−−) indicates that physical factors but not predation or feeding hypotheses explain the observed molting or reproductive rhythm in conjunction with the depth distribution of the species.

G. zacae occurs in coral rubble from low, occasionally exposed intertidal areas to at least 70 meters in depth; this species molts (+−−) and probably reproduces (+−−) during "neap" tides. *G. falcatus* occurs in moderately low intertidal coral rubble in Hawaii (exposed by moderate spring tides), in the low intertidal in Australia (exposed by low spring tides), and in the very low intertidal in the Marshall Islands, the Gulf of Thailand, and the Andaman Sea (where exposure occurs on very low spring tides). *G. falcatus* tends to molt during spring tides in Hawaii (−+−), Australia (−+−), and Thailand (−+−), and oviposits during "neap" tides in Hawaii (+−−). *P. ciliata* occurs under and in coral rubble and vegetation over a fairly wide shallow subtidal and low intertidal habitat, but is most abundant in moderately high intertidal areas in Hawaii, Australia and Thailand. *P. ciliata* molts during "spring" tides (+−+), and oviposits (Hawaii and Florida) during "neap" tides (−+−). In Australia, five species of *Gonodactylus* occur in the low intertidal coral rubble which is exposed only during low spring tides. Although the other four species apparently are restricted to the low intertidal and shallow subtidal, *G. graphurus* occurs as deep as 30 meters. These species do not show molting rhythms (−−+) but oviposit during spring tides (−+−). In Thailand, *G. ternatensis* is restricted to the extremely low intertidal coral and is rarely exposed. *G. falcatus* is exposed regularly, although briefly, by very low spring tides. *P. ciliata, G. smithii*, and *G. viridis* all co-occur with *G. falcatus* but extend into shallow intertidal areas as well. *H. glyptocercus* occurs from mid-intertidal coral rubble to high intertidal rock. *G. viridis* extends to high intertidal coral and rock, encountering extensive exposure during most low tides. *G. chiragra* extends upward from the moderately low intertidal rubble but predominates in the high intertidal rock, which is exposed for longer periods during neap than spring tides. *G. chiragra* molts significantly more frequently than expected during spring than neap tidal cycles in the field (+−+), as do all of the species of stomatopods in Thailand (−+−). Oviposition occurs during spring tides among these species (−+−).

Selection by physical factors correctly predicts the tidal cycle for molting or reproduction, given the species distribution, in five of thirteen cases; selection by predation explains seven of thirteen cases; and selection by feeding time explains three of thirteen cases. In the cases discussed above, molting and reproduction occur during the same tidal cycle in two of five cases. These data provide poor evidence that selection from physical factors, predation, or feeding time favors molting and reproductive synchrony.

The fourth explanation for the selective advantage of semi-lunar or tidal molting and reproductive synchrony, the *selfish herd hypothesis*, predicts that peaks of molting and reproduction may occur in either spring or neap tidal cycles in different populations from different geographical areas; that one population probably will show the same rhythmicity in high, low and subtidal areas, but will not necessarily molt and oviposit during the same tidal phase; and that different species within a sympatric stomatopod community will show similar molting and reproductive rhythms. These predictions are mostly confirmed by the above data.

Hamilton's (1971) selfish herd hypothesis proposes that some social aggregations may result from avoidance of predators by individuals which hide among others of their own species. The present hypothesis for tidal molting synchrony is a temporal modification of the selfish herd hypothesis. Individuals which molt when most other individuals are also molting may suffer low mortality from aggressive congeneric and conspecific stomatopods.

Fighting and the hazards of molting are accentuated even early in ontogeny among stomatopods. Larval instars of *Squilla* attack, kill, and sometimes eat conspecifics, especially when the latter are molting (Alikunhi, 1950; Pyne, 1972). This aggressive behavior intensifies over ontogeny (Reaka, in preparation). If maintained communally, adult squillids and gonodactylids will kill and frequently devour a molting conspecific or congeneric. Individuals in the late premolt and early postmolt stages exhibit decreased dominance; recently molted individuals suffer extensive damage and frequent mortality during fights or handling. Displacement from a burrow by a conspecific or congeneric and exposure to predation may be even more dangerous than fighting injuries. Vulnerability at the molt is undoubtedly one of the selective pressures which favors plugging of the cavity entrance(s) and burrowing behavior as the molt approaches (Reaka, 1975a).

An advantage to the individual accrues if it is entrained genetically and/or environmentally to molt about the same time as the rest of the population. For an extreme example, consider that in a population of 100 animals, 99 molt within one lunar phase. Any individual that does not molt and remains out of phase with the population could invade, perhaps eat, and occupy the cavity of any of the 99 molting individuals in a species which does not seal the burrow or otherwise hide at molting. The probability that one of the molting individuals will be displaced or killed is only 1/99. However, when the asynchronous individual subsequently molts, the probability that it may be displaced or killed by the other 99 intermolt individuals is 99/100. A similar system would apply to the bimodal pattern established in *G. zacae,* for example, but the margin of advantage would be smaller. If it is assumed that a constant number of individuals which molt are killed by aggression (*e.g.,* five during each of the four lunar phases), then it is obvious from Table I that an individual which molts during I or L would be selected against, since the probability of being killed would be 5/47, 5/19, 5/47, and 5/31 in the four lunar phases, respectively. In fact, the probability than an asynchronous individual will be killed is even higher because fewer individuals are molting during I and L, and therefore more voracious intermolt stomatopods are available to effect a greater number of killings during I and L (*e.g.,* 5/47, 8/19, 5/47, 8/31 or 11%, 42%, 11%, and 26% chance of mortality at the molt). Therefore, once initiated in the population, selection for molting synchrony should be a positive feedback spiral.

Because of low molting rates, a relatively small percentage of the population molts at a given time. However, natural selection should focus upon this segment of the population, favoring reception of cues which initiate molting at a time when most other individuals molt. Stomatopods (as well as lobsters and crabs) possess long mid- or subterminal stages of the molt cycle, followed by rapid completion of the stages preceding the molt (Reaka, 1975a). These observations suggest that an individual may wait in subterminal stages of the molt cycle for a considerable time before rapidly completing the molt upon reception of appropriate stimuli. An individual may cheat, or pillage the surroundings during cycles of molting in the population, then molt in phase with the population. In fact, the population may be comprised of reciprocally cheating individuals. However, this only accentuates the advantage to an individual which responds opportunistically to cues, hides and/or seals its cavity, and molts when the maximum number of other individuals also are molting. This type of cheating is probably a natural consequence of intermolt intervals which span a number of peaks of molting in the population, and should not cause a collapse of the system of molting synchrony because each molting individual should still scramble to get to "the middle of the herd."

One effect of molt synchrony would be to provide a phasic sequence of predation by stomatopods and introduction of instability into the predator-prey environment. Johnson (1973) discusses phasic predation resulting from molt synchrony of damselfly naiads preying upon cladocerans. In stomatopods one would expect selection against molting synchrony if food limits the population; molting synchrony should introduce periods of increased and decreased competition for food among the predators. Population density of the stomatopods would be limited by the periods of high competition food when few stomatopods are molting. Asynchronous molting would effect an average but stable level of competition for food, and density equilibrium of the stomatopods would be higher than above. Also, populations of the prey species would be maintained at low densities by the peak feeding phases in the intermolt cycle of a molt-synchronized predator. Populations of prey species might be extinguished by intense phasic predation, lowering prey diversity as well. Phasic molting and predation, even on a predictable basis, would probably break down in a population of stomatopods limited by food. Alternatively, if congeneric and conspecific competition for burrows (rather than food) limits populations of stomatopods, then molting synchrony is one of several possible adaptations which could decrease mortality associated with molting.

The initiation of molting synchrony in a population is more difficult to explain than the maintenance of this trait. Local populations, nondispersive larval behavior, and synchronous release and/or hatching of eggs could initiate molting synchrony. Several kinds of evidence suggest that these phenomena occur in gonodactylid stomatopods. *G. falcatus* and *G. zacae* are highly color polymorphic. Localized demes are uniform in body size and color relative to those on other patch reefs. Although *G. falcatus* was introduced to Oahu approximately 30 years ago, has increased markedly in density, and has displaced another gonodactylid (Kinzie, 1968), it has not colonized adjacent outer islands of the Hawaiian chain except for Molokai, in spite of available habitat on the other islands (personal observations; R. A. Kinzie, University of Hawaii, personal communication; W. VanHeukelem, University of Hawaii, personal communication). The behavior of larval stages of *Squilla* and *Lysiosquilla* keeps them near the substrate and in dark places (Alikunhi, 1950; Pyne, 1972). Gonodactylids possess fewer "pelagic" stages than squillids (Gohar and Al-Kholy, 1957). Although larvae of *G. bredini* reportedly become photopositive after the fourth instar (Dingle, 1969), other observations suggest that swimming activity is not sustained and therefore that larval dispersal may not be extensive (Pyne, 1972), particularly in the gonodactylids (personal observations). Therefore, molting synchrony could be established by chance, and maintained by selection as discussed above, in small nondispersive populations. Also, oviposition rhythms may initiate molting rhythms. Synchronous hatching probably results from synchronous oviposition. Since synchronous hatching precedes synchronous molting and development in isopods, it is possible that the larvae of stomatopods develop synchronously without extensive dispersal, and therefore initiate local molt synchrony where they settle. Reproductive synchrony could evolve in response to other factors, such as availability of food for or predation upon larvae, or finding a receptive mate in a population where few individuals are reproductive at any one time. In addition, however, it seems likely that selection would favor those individuals which reproduce in a lunar or tidal phase and the young of which molt in phase with the population.

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SUMMARY

1. During laboratory maintenance, *G. zacae* from the Gulf of California molts during neap tides or the transition from spring to neap tides; oviposition probably occurs during neap tides or the transition from spring to neap tides. *G. falcatus* from Hawaii tends to molt during spring tides or the transition from neap to spring tides; oviposition occurs during neap tides or the transition from spring

to neap tides. Strong evidence suggests that *H. glyptocercus* from Eniwetok molts during neap tides or their approach. *P. ciliata* from three populations molt during the last lunar phase, providing the only substantive evidence for lunar rather than tidal control of molting in stomatopods. While *P. ciliata* from Hawaii and Florida lays eggs during neap or waxing neap tidal phases, those maintained in Thailand deposit eggs during waning neap tidal cycles. *G. falcatus* and *G. graphurus* from Australia molt in opposite tidal cycles; *G. graphurus* molts during neap tides but reproduces during spring tides. The five species of Gonodactylidae from Australia oviposit during spring tides.

2. Field data demonstrate significantly more molting during spring than neap tidal cycles in a gonodactylid community comprised of seven species in Phuket, Thailand. Laboratory maintenance increases variation in the pattern of molting across tidal cycles, although molting still occurs significantly more frequently in spring than neap tidal cycles. *G. chiragra* molts significantly more frequently than expected during spring than neap tides in the field. Both field and laboratory data demonstrate significant correlation of oviposition with spring tides among the species in 1973 but not 1974.

3. These data support a temporal selfish herd hypothesis that synchrony of molting in the population reduces mortality due to conspecific and congeneric aggression in stomatopods. Observed data do not support predictions from alternative hypotheses that physical factors, feeding, or predation impose molting and reproductive rhythms. Molting synchrony is more consistent with the idea that stomatopod populations are limited by the availability of burrows than by food. The initiation of molting synchrony in the population can be explained by selection for response to environmental cues, by chance, and/or by small, local populations with synchronous hatching of young. Oviposition rhythms therefore may subserve the primary molting rhythm.

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HATCHING SIZE AND THE DISTRIBUTION OF NURSE EGGS AMONG PROSOBRANCH EMBRYOS [1]

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A prosobranch embryo that feeds on nurse eggs will often reach a hatching size much larger or smaller than the mean for its species. In contrast, an embryo is likely to reach a standard size when its entire yolk supply is enclosed within its egg membrane (Fioroni, 1966). A variable hatching size may be an appreciable disadvantage (for example, if there is an optimum hatching size; Smith and Fretwell, 1974). Therefore, the factors leading to hatching size variations are likely to be of substantial importance to the snails.

An individual embryo can attain a relatively large hatching size only if it is able to acquire more than the average number of nurse eggs during its feeding period. An unequal distribution of nurse eggs can be obtained by three means. First, nurse-egg supplies may vary from egg capsule to egg capsule; when one capsule is more well-stocked with nurse eggs than others are, each of its embryos will acquire more than the average nurse egg supply. Second, some capsules may contain many more embryos than others even though all capsules are about equally well stocked with nurse eggs. Third, some embryos within each capsule may obtain more than their share of the nurse egg supply, thus attaining large size at the expense of (*e.g.*, out-competing) their capsulemates. The present research was initiated to determine which of these means is most important for two Eastern Pacific rocky shore snails, *Thais emarginata* and *Acanthina spirata* (Prosobranchia: Muricidae).

MATERIALS AND METHODS

To obtain egg and embryo counts, capsules and adults of *Thais emarginata* were collected near Friday Harbor, San Juan Island, Washington, and from Dillon Beach (Bodega Bay) and Tomales Bay (Miller Park), Marin County, California; and capsules and adults of *Acanthina spirata* were collected from Tomales Bay. Females deposited capsules in the running water aquaria in both Washington (Friday Harbor Laboratories) and California (Pacific Marine Station). Field and laboratory capsules were kept in the aquaria until analyses were complete.

To obtain hatching sizes, capsules were isolated in small, closed containers during the hatching period. Hatchlings of *T. emarginata* were obtained unsystematically at Friday Harbor but all individuals of both species that hatched in the containers at Dillon Beach were measured.

Actual hatching sizes can be obtained only at the time of hatching. To obtain large samples on demand, an alternative measure was utilized—the sizes of late embryos (embryos that withdraw fully into their protoconchs). In California, the average late embryo of *T. emarginata* reached a shell length (1.195 mm; N, 133; s.d., 0.176) nearly the same as that of a newly hatched snail (1.182 mm; N, 27; s.d., 0.141). No hatchlings were smaller than 0.9 mm (although some late embryos were), and 0.9 mm is accepted as a minimum hatching size. Late embryos of *A. spirata* are smaller (0.635 mm; N, 14; s.d., 0.029) than newly hatched snails (0.671 mm; N, 26; s.d., 0.058). Late embryo sizes cannot be equated precisely with hatching sizes, but they will be used as convenient and reasonable approximations.

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Nurse-egg rations for *A. spirata* were obtained by comparing counts taken at cleavage stages (eggs + embryos) with counts of late embryos (embryos only). Larger capsules generally contain more embryos than smaller capsules (Spight, Birkeland and Lyons, 1974), and therefore, one must allow for capsule size differences when making comparisons. To do this, a relationship between capsule size and egg or embryo count was estimated for each sample of capsules. Linear relationships were obtained by regressing logarithms of counts on logarithms of capsule lengths (capsule body length, excluding stem). The counts included small and aberrant embryos, but data for capsules with excess nurse eggs were omitted (these capsules presumably did not have full complements of embryos).

Egg counts of *T. emarginata* were not made, but were instead estimated from late embryo sizes. The volume attained by an embryo was assumed to be proportional to the volume of yolk it consumed. A "volume index" proportional to the initial yolk volume was obtained for each capsule by adding the logarithmic mean embryo size to the logarithm of the number of embryos. The necessary data (sizes of all embryos) were obtained for twelve normal capsules and ten capsules with excess nurse eggs.

RESULTS

Variability of hatching size

Newly hatched *Thais emarginata* from Friday Harbor had shell lengths of 1.55 to 1.80 mm; ones from Tomales Bay were 0.87 to 1.38 mm long (mean, 1.182 mm; s.d., 0.141; N, 27); and ones from Dillon Beach were 1.00 to 1.55 mm long (mean, 1.330 mm; s.d., 0.176; N, 10). Newly hatched *Acanthina spirata* from Tomales Bay were 0.55 to 0.75 mm long (mean, 0.67 mm; s.d., 0.058; N, 26).

Nurse-egg rations: Acanthina

All eggs of *Acanthina spirata* form gastrulae but some do not develop further, and the arrested eggs are eaten by the embryos. Some capsules from Tomales Bay (July 1973) contained 40–140 eggs in early cleavage stages, while other capsules contained 17–46 late embryos (Fig. 1). The mean number of late embryos (29.6; s.d., 7.84; N, 13) was subtracted from the mean number of eggs (79.0; s.d., 26.39; N, 11) to obtain the number of nurse eggs (on average, 1.67 nurse eggs per embryo; filled symbols of Fig. 1).

Both egg and embryo counts increase in proportion to capsule size (Fig. 1). To quantify the significance of the increase, coefficients of determination (r^2) were

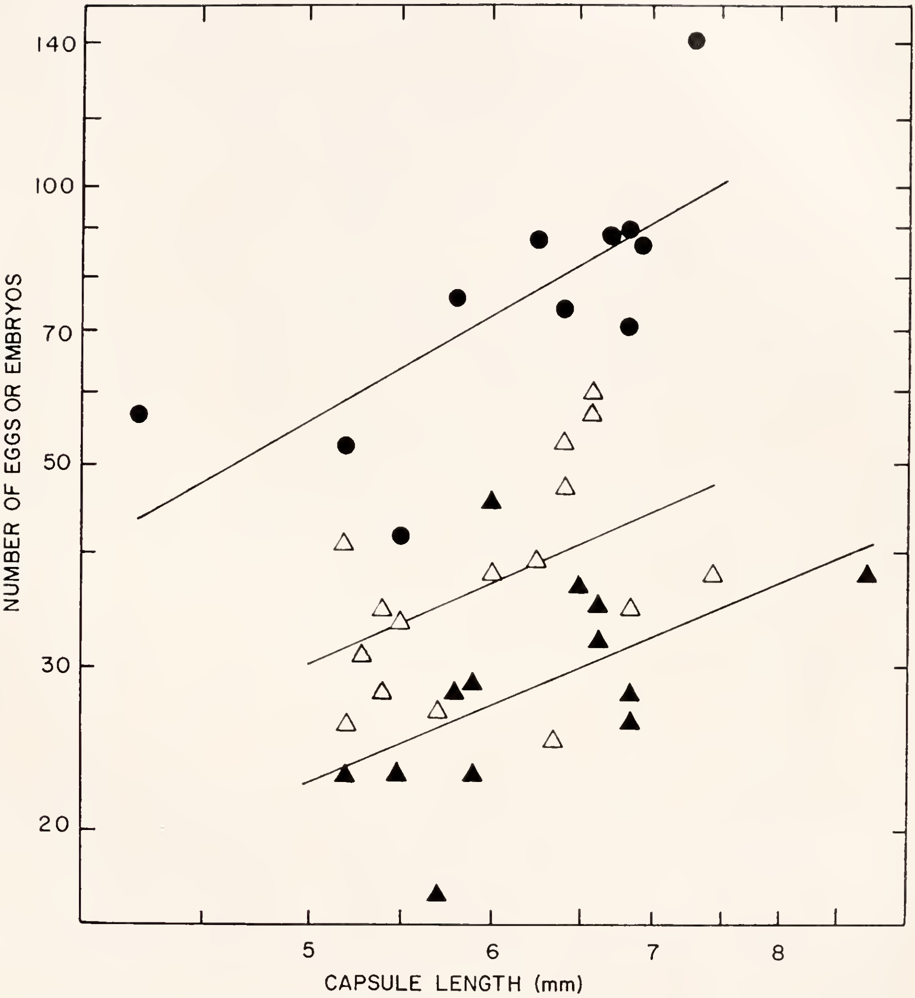

FIGURE 1. Contents of capsules of *Acanthina spirata* that were collected from the field (Tomales Bay, California, filled symbols), or were deposited in the laboratory (open symbols). Capsules containing egg stages (embryos plus nurse eggs) are indicated with circles, and capsules containing late embryos are indicated with triangles. Least-squares regression lines are plotted. Note that both scales are logarithmic.

calculated for these paired variables (Table I). Capsule length accounts for 55% of the variations in egg counts (r^2, 0.55). However, capsule size accounts for only 25% to 28% of the variations in embryo counts.

Several capsules were deposited in the laboratory, and these contained more embryos (38.4; s.d., 10.88; N, 16) than capsules from the field (Fig. 1). The late embryos in these capsules were also significantly smaller (mean shell length, 0.635 mm; s.d., 0.029; N, 14 embryos) than those found in capsules from Tomales Bay (mean, 0.699 mm; s.d., 0.049; N, 24; $F_{1,36} = 18.94$, $P < 0.001$).

TABLE I

Least-squares regression of logarithms of the number of embryos (Y) on logarithms of capsule length (X, mm). Capsules were collected in the field (F) in California and Washington, or were deposited in running-water laboratory aquaria (L). Egg counts (last line only) include both nurse eggs and embryos. All other counts include only late veligers. The total for Thais emarginata *includes four capsules collected at Dillon Beach.*

Source	Relationship	Number of capsules	Coefficient of determination	Mean square error	$F_{1,N-2}$	$\bar{X}$
	Thais emarginata					
Tomales Bay (F)	$Y = 2.2590X - 0.7168$	14	0.210	0.0431	3.1927ns	0.8362
Tomales Bay (L)	$Y = 1.5110X - 0.0402$	19	0.254	0.0252	5.7741*	0.7818
Study Strip	$Y = 2.3473X - 0.8007$	17	0.398	0.0215	9.9232**	0.8076
Shady Cove	$Y = 2.5889X - 0.8506$	9	0.087	0.0180	0.6656ns	0.7787
Total	$Y = 1.6360X - 0.1683$	63	0.202	0.0295	15.4101**	0.7989
	Acanthina spirata					
Veligers (F)	$Y = 1.0619X + 0.6115$	13	0.282	0.0105	4.9200ns	0.7967
Veligers (L)	$Y = 1.2541X + 0.5934$	16	0.250	0.0113	4.6692*	0.7775
Eggs	$Y = 1.4714X + 0.7210$	11	0.546	0.0100	10.8230**	0.7855

* $P < 0.05$; ** $P < 0.01$; ns, not significant.

Based on the egg counts for the field capsules, each laboratory embryo had 1.06 nurse eggs.

Nurse-egg rations: Thais

Large capsules of *Thais emarginata* generally contain more nurse eggs and more embryos than smaller ones. Capsule length (X) accounts for 54% of the variations in egg content (Y), when the volume index is used as a measure of egg content (nurse eggs + embryos). The linear regression between these two variables is: $Y = 2.3513\ X + 2.3082$, for twelve capsules. Embryo counts (Fig. 2) are more variable; capsule length accounts for only about 20% of the variations in embryo count, and a unique relationship was obtained for each of the samples (Table I).

When embryo counts are highly variable, occasional large capsules will contain a few embryos and many nurse eggs, while occasional small capsules will contain many embryos and few nurse eggs. Maximum and minimum counts were examined to determine likely limits to nurse egg rations. Maximum counts increase steadily with capsule size. Among the capsules opened, 15 were smaller than 5.6 mm and none contained more than 20 embryos, while 85 capsules were longer than 5.6 mm and 18 of them contained 21 to 33 embryos. Within a length range of 4.3 to 8.2 mm, only capsules 6.2 to 7.7 mm contained 30 or more embryos, and the 7.7 mm capsule contained the maximum number of embryos observed (33). On the other hand, minimum counts do not increase with capsule size. In total, 11 capsules contained only 1 to 4 embryos, and 6 of these were larger than the average (6.3 mm; s.d., 0.77; N, 63). Therefore, the same minimum embryo count is common to capsules of all sizes.

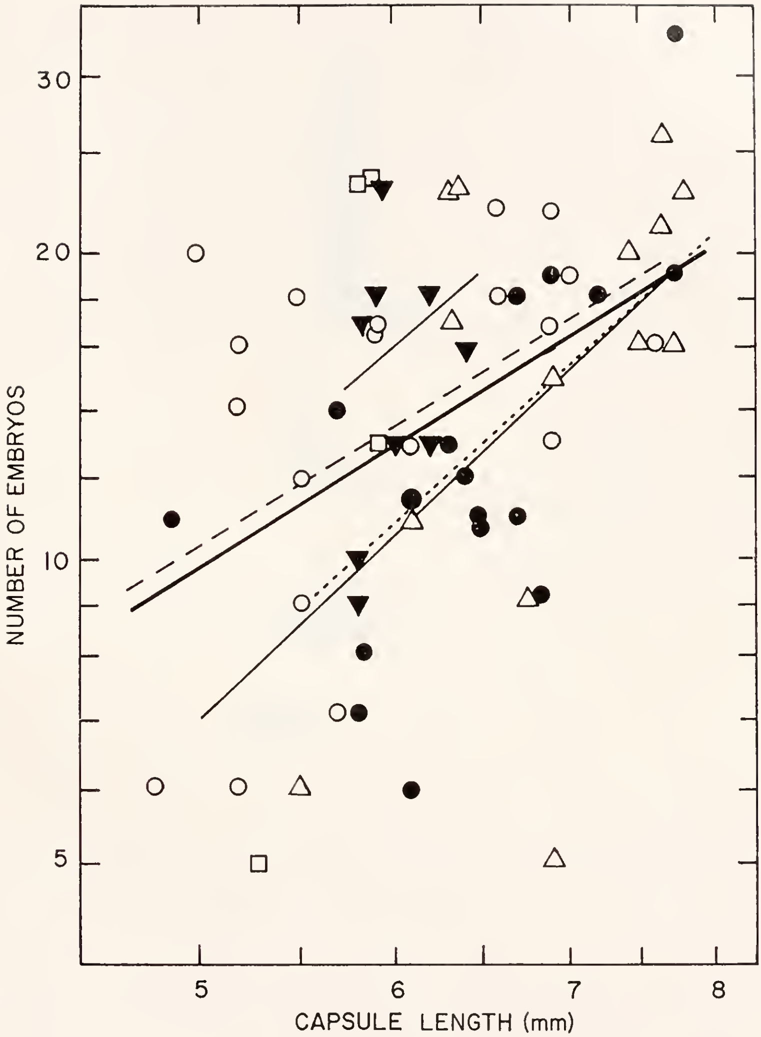

FIGURE 2. Contents (number of late embryos) of capsules of *Thais emarginata* that were collected in Washington (filled symbols) and California (open symbols). Washington capsules were obtained at two localities, Shady Cove (inverted triangles and upper solid line) and Study Strip (circles and lower solid line; see Spight, 1974). California capsules were obtained at Tomales Bay (field capsules, triangles and dotted line; laboratory capsules, circles and dashed line) and at Dillon Beach (squares). The heavy line in the center is a least-squares regression including all capsules.

Hatching size and number of capsulemates

Embryos have usually eaten all their nurse eggs long before the developmental period ends. However, some nurse eggs are left over when capsules of *T. emarginata* contain only one to four embryos. The volume indices (Y) for ten

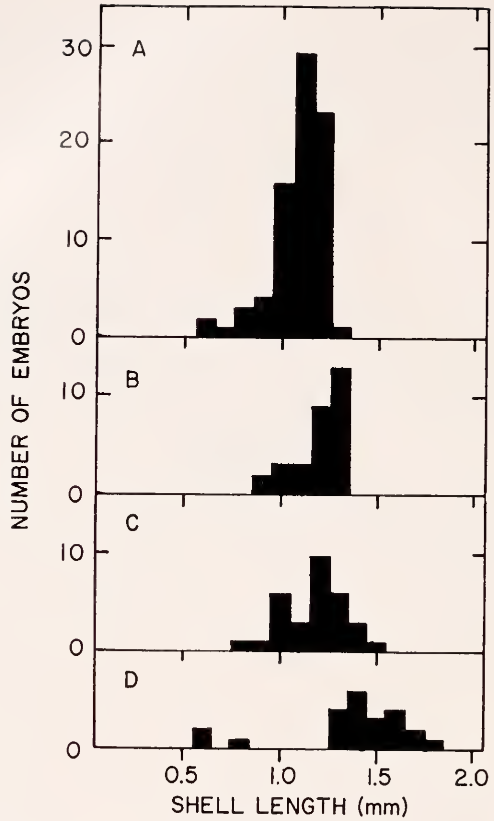

FIGURE 3. Effect of crowding on sizes reached by embryos of *Thais emarginata*. Capsules in A contain 17–23 embryos; capsules in B contain 14–16 embryos; capsules in C contain 6–11 embryos; and capsules in D contain 1–4 embryos (and excess nurse eggs). In a capsule containing four embryos, one embryo reached 3.02 mm; its size is not plotted.

such capsules are highly correlated (r^2, 0.90) with the number of embryos (X), indicating that all embryos eat about the same number of nurse eggs when nurse eggs are present in excess. With the relationship between these variables, $Y = 0.2066X + 2.9683$, it can be shown that the nurse egg supplies of the smallest capsules can be consumed by only four embryos, and six embryos can consume the supplies found in the largest capsules.

Since nurse egg supplies vary less from capsule to capsule than embryo counts, a typical embryo's nurse-egg ration will be more closely related to the number of capsulemates than to capsule size. In the capsule sample available, embryo sizes (Y, mean of the logarithms of shell lengths in mm) are highly correlated (r is −0.69) with embryo counts (number per capsule, X). The linear regression between these variables is $Y = 3.1463 - 0.0058\ X$, for twelve capsules.

Embryo size distributions, as well as mean sizes, change regularly as the number of embryos per capsule decreases (Fig. 3). When 17–23 embryos share a capsule, the distribution is sharply curtailed to the right and has a small tail to the left (Fig. 3A). Most embryos reach about the same size. Only 11 of 79 fall outside the narrow range, 1.0–1.2 mm; of these, six are smaller than the minimum hatching size of 0.9 mm, and only one has reached 1.3 mm. When 14–16 embryos share a capsule, the distribution is still curtailed to the right, but the left-hand tail contains a larger proportion of the embryos (Fig. 3B). The modal size increases to 1.3 mm, but no embryos have reached 1.4 mm (all are larger than 0.9 mm). With only 6–11 embryos, sizes form a nearly symmetrical distribution (Fig. 3C). Some embryos reach 1.5 and 1.8 mm, and others fail to reach 0.9 mm. When only 1–4 embryos share a capsule, the size distribution is nearly a mirror image of those for the crowded capsules. The distribution tails off to the right, and except for the three very small embryos, is sharply curtailed to the left (Fig. 3D).

Discussion

When a species provides nurse eggs to its offspring, some embryos typically reach much larger sizes than others. For example, hatching sizes range from 0.54 to 1.83 mm for *Murex virgineus* (Natarajan, 1957), 0.3 to 2.5 mm for *Thais lapillus* (Risbec, 1937), 3.5 to 8.5 mm for *Sipho islandicus* (Thorson, 1935), and 0.7 to 1.8 for *Murex brandaris* (the most variable of the species studied by Fioroni, 1966). Embryos of *Thais emarginata* and *Acanthina spirata* are provided with nurse eggs and reach variable hatching sizes. *T. emarginata* hatches as small as 0.9 mm and as large as 1.8 mm, a range of +44% to −31% about the mean shell length, and an 800% variation in weight. Hatching sizes for *Acanthina spirata* vary from +15% to −16% about the mean size, a range typical for species without nurse eggs (Fioroni, 1966).

An embryo must acquire more than the average nurse-egg supply to become larger than average. If excess nurse eggs are available, these will be eaten readily; embryos are usually capable of consuming several times the normal supply. For example, embryos of *Murex quadrifrons* and *Sipho curtis* will consume two to three times their usual rations (Knudsen, 1950; Thorson, 1935). The largest capsules of *T. emarginata* contain only enough nurse eggs to satiate six embryos. Since capsules rarely contain as few as six embryos (Fig. 2), typical embryos would eat many more nurse eggs than they get.

Since nurse eggs are in short supply, competition among capsulemates may affect hatching sizes. An embryo that begins feeding early and feeds rapidly will acquire more nurse eggs than a capsulemate that begins feeding late and feeds slowly. The size distributions for *Thais emarginata* show that some embryos acquire many more nurse eggs than their capsulemates. Embryos as small as 0.8 mm were found together with others as large as 1.8 mm after all nurse eggs had been eaten. However, large size differences among capsulemates are not typical (Fig. 3). When many embryos share a food supply, most reach about the same size; intracapsular size ranges in crowded capsules are as small as 1.0–1.2 mm. If competitive ability affected hatching size appreciably, then capsulemates would tend to be most dissimilar when food supplies are smallest (*e.g.*, in the most crowded capsules), and all embryos would tend to reach the same size when nurse eggs are present in excess. However, exactly the opposite is observed; embryo sizes are most varied when nurse eggs are present in excess (Fig. 3D; note the gradual increase in coefficient of variation of the mean: 13% for 17–23 embryos; 11% for 14–16 embryos; 16% for 6–11 embryos; and 23% for 1–4 embryos, excluding the three very small embryos of Fig. 3D). Therefore, competitive ability does not play a major role in generating hatching size differences among embryos of *T. emarginata.*

If competitive ability does not determine how nurse eggs are allocated, then nurse-egg rations must vary from capsule to capsule. The average embryo is substantially larger in some capsules than in others (Fig. 3). Therefore, nurse-egg rations are substantially larger than the average in some capsules.

An embryo will have a large nurse-egg ration if its capsule contains more than the usual number of nurse eggs, or, alternatively, if its capsule contains fewer than the usual number of embryos. Total egg counts (embryos plus nurse eggs) do vary from capsule to capsule. However, the counts are closely related to capsule size (Fig. 1); for *Acanthina spirata* and *Thais emarginata,* capsule size accounts for 54–55% of the variations in total egg count. Similar correlations between total egg counts and capsule sizes are observed for species without nurse eggs (five species: r^2 is 0.55 to 0.94, except *Eupleura caudata,* in which r^2 is 0.22; Spight, 1972; Spight, Birkeland and Lyons, 1974). Embryo counts are less predictable. Although small capsules seldom contain many embryos, some of the largest capsules contain only one or two embryos, and, overall, correlations between capsule size and embryo count are only marginally significant (Table I). Since embryo counts vary erratically from capsule to capsule, and nurse egg counts do not, embryos in some capsules will share a nurse egg supply with many capsulemates, while embryos in other capsules will share with few capsulemates. Each of the latter embryos will obtain a relatively large nurse egg ration and reach a large hatching size.

These data demonstrate that hatching sizes are variable for nurse-egg feeders because embryos are distributed erratically among capsules. Further work will be required to determine why embryos are distributed erratically and what advantages nurse-egg feeding provides to compensate for the disadvantage of a variable hatching size.

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SUMMARY

Some embryos of *Thais emarginata* attain twice the shell length of others by the time of hatching (0.9 to 1.8 mm, +44% to −31%, about the mean). Larger hatchlings must have acquired more nurse eggs than smaller ones. Embryos tend to reach the same size when nurse eggs are scarce (in crowded capsules), and size differences are most apparent when nurse eggs are present in excess. Therefore, competition among embryos contributes little to hatching size differences. Embryos are distributed haphazardly among capsules; some capsules contain only one embryo which becomes large, while others contain as many as 33 embryos, each of which

remains small. Nurse eggs are distributed more regularly. Embryos are also distributed haphazardly among capsules of *Acanthina spirata*. Each embryo of *A. spirata* has on average only 1.67 nurse eggs, and hatching size is correspondingly less variable (0.55–0.75 mm, +15% to −16%, about the mean). These nurse-egg feeders reach variable hatching sizes because some embryos share their yolk supplies with many more capsulemates than others.

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ACTIVATORY MECHANISMS OF THE PROTHORACIC GLANDS OF *MONEMA FLAVESCENS* (LEPIDOPTERA) WITH SPECIAL REFERENCE TO THE SECRETION OF ECDYSONE

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It had been assumed that the prothoracic glands synthesize and secrete ecdysone in the manner of an endocrine gland, despite the lack of direct experimental evidence, until a first preliminary report that the prothoracic glands can secrete ecdysone *in vitro* appeared (Takeda, 1972a). This classical theory came basically from the experimental morphological findings that the insects into which active prothoracic glands were implanted were induced to molt (Fukuda, 1940, 1944). The amount of secreted hormone has always been too small to provide proof that this gland is the site of ecdysone synthesis.

It is well known that α-ecdysone was isolated first from *Bombyx mori* (Butenandt and Karlson, 1954), synthesized from cholesterol (Karlson and Hoffmeister, 1963; Galbraith, Horn, Middleton, Thomson and Thomson, 1970; Moriyama, Nakanishi, King, Okauchi Siddail and Hafferl, 1970; Gersch and Stürzebecher, 1971; Willig, Rees and Goodwin, 1971; Nakanishi, Moriyama, Okauchi, Fujioka and Koreeda, 1972; Studinger and Willig, 1975) and is rapidly metabolized to β-ecdysone (King and Siddall, 1969; Moriyama *et al.*, 1970; Nakanishi *et al.*, 1972). The possibility was reported that the prothoracic glands secrete into the hemolymph an enzyme necessary for ecdysone biosynthesis from cholesterol (Gilbert, 1964; Bern, 1967). Locke (1969) and Weir (1970) reported that ecdysone may be secreted from the oenocyte. Further, Romer (1973) extracted a considerable amount of ecdysones from isolated oenocytes of *Tenebrio molitor*. Moriyama *et al.* (1970) found α-ecdysone synthesis in the abdomen of *Bombyx mori* which did not contain the prothoracic glands. They postulated that the prothoracic gland hormone was not identical to ecdysone but acts to transform the inactive-bound ecdysone to the active form. Therefore, a possibility arises that the prothoracic glands are the source of a second trophic hormone rather than ecdysone. Since no prothoracic glands are present in the isolated abdomen, a doubt is cast on the long standing principle in insect endocrinology that the prothoracic glands are the source of ecdysone synthesis. Nakanishi *et al.* (1972) postulated strongly that the prothoracic glands act only as a source of an enzyme necessary for the biosynthesis of ecdysone from cholesterol.

Recent *in vitro* work, however, supports the original notion that the prothoracic glands do synthesize ecdysones or substances with ecdysone-like activity. Willig *et al.* (1971) reported that isolated ring gland-brain complexes of the blowfly, *Calliphora erythrocephara,* incubated with cholesterol, synthesized ecdysone, most often in the form of glycoside and esters. It was also found that spermatogenesis in the isolated testes of diapausing silkworm pupa, *Hyalophora cecropia,* incubated in a cell-free hemolymph was stimulated *in vitro* by the addition of activated prothoracic glands or by inactive glands together with active brain or ecdysone. However, inactive glands or active brain alone produced no response (Kambysellis and Williams, 1971, 1972). It was ascertained that the prothoracic glands taken from six-day old larvae in the final instar of the cabbage armyworm, *Manestra brassicae,* released a hormonal agent which induced the molting of the integument of the rice stem borer, *Chilo suppresalis* (Agui, Kimura and Fukaya, 1972). More recently, other research groups have provided some chemical evidence that the cultured prothoracic glands of the silkworm, *Bombyx mori* (Chino, Sakurai, Ohtaki, Ikekawa, Miyazaki, Ishibashi and Abuki, 1974), the tobacco hornworm, *Manduca sexta* (King, Bollenbacher, Borst, Vedeckis, O'Connor, Ittycheriah and Gilbert, 1974), the cockroach, *Leucophaea maderae* (Borst and Engelmann, 1974; King and Marks, 1974) and the mealworm, *Tenebrio molitor* (Romer, Emmerich and Nowock, 1974) produce α-ecdysone.

Although the physiological properties of ecdysone have been extensively studied (Novák, 1966; Šorm and Sláma, 1974), the synthetic site of ecdysone has not been clear. It waxes and wanes under the direction of the central nervous system which intermittently releases a tropic hormone referred to as the prothoracotropic hormone (brain hormone), and finally regresses following the terminal molt on the diapause break (Fukuda, 1944; Williams, 1947, 1952). In *Monema flavescens,* the neurosecretory substances, the prothoracotropic hormones, are released from the neurosecretory B-cells in the *pars intercerebralis* (Takeda, 1972b). These substances migrate directly across the perineurium to the hemolymph (Takeda, 1976). Then, they are transported to the prothoracic glands by the granular hemocytes and activate them (Takeda, 1971a).

On the other hand, many papers on the histology of the prothoracic glands have appeared (Herman, 1967). However, knowledge of the prothoracic glands in relation to the secretion seems to be small (Beaulaton, 1964, 1968; Herman and Gilbert, 1966; Yashika and Yoshizaki, 1967). For these reasons, the problems of secretion in the prothoracic glands with special reference to ecdysone synthesis are investigated in this study.

Materials and Methods

Materials used in this study were the slug moth prepupa, *Monema flavescens* Walker (Lepidoptera: Heterogeneidae). Specimens were collected at the diapause period in the field and placed under natural conditions. When each experiment was carried out, parts of the materials were incubated in order to estimate the degree of diapause intensity (Takeda, 1971b).

For light-microscopic studies, the head regions were fixed with Bouin, Susa, Susa-picric and Kahle's methods modified by Huttner for 24 hours, respectively, at intervals from September to the following March. They were dehydrated in butyl alcohol, embedded in paraffin wax and sectioned at 3 μ. Staining methods applied in these experiments were Hematoxylin-eosin, Mallory's triple stain, Gabe's chromalum hematoxylin phyloxin, Clark's aldehyde fuchsin and $HgCl_2$ bromphenol blue.

For electron-microscopic studies, the prothoracic glands were quickly extirpated under a dissecting microscope, then immediately pre-fixed in cold 2.5% glutaraldehyde (pH 7.4: phosphate buffer) for ninety minutes and were post-fixed in cold 2% osmium tetraoxide (pH 7.4: Veronal buffer) for three hours. They were passed through cold 50%, 70%, and 90% ethyl alcohol for 45 minutes at room temperature. After dehydration, they were placed in absolute acetone for one hour and were gradually infiltrated with Epon 812 (Luft, 1961). The Epon blocks were trimmed and sectioned using an ultramicrotome with glass knives. The staining was performed with lead citrate (Reynolds, 1963). Micrographs were taken with JEM-T6S electron microscopy.

For autoradiography, tritiated uridine was employed at a concentration 1 mCi/mM with a specific activity 1.9 mCi/mM. All insects were killed several hours after the injection. They were embedded in paraffin wax and cut at 3 μ. Serial sections were coated with Sakura nuclear-track emulsion, NR-M2. The coated slides were exposed for four weeks at 0° C. All slides were developed at 20° C for one minute in Konidol X (Sakura). Autoradiographs were stained with Mayer's hemalum-fast green.

Histochemical tests for cholesterol were carried out in the prothoracic glands fixed with formalin. The tissues were then treated with 0.5% digitonin in ethyl alcohol (50%) for ten days, and then with ethyl alcohol and ether for three hours. After that, Lieberman-Schultz's methods for cholesterol were applied to the frozen sections obtained by cryostat.

For another autoradiography, ^{14}C-cholesterol (^{14}C-cholesterol-4-C: specific activity, 33.5 mCi/mM) was employed at a concentration of 1 mCi/ml. A dosage of 1 μCi/1 g of body weight was injected into the abdomen with a microsyringe. After several hours (12, 24 and 48 hours), insects were fixed with Bouin containing saturated digitonin. Digitonin is known to make an unsoluble complex in some organic solvents reacting with isolated cholesterol. Therefore, this treatment is known to make it easy to capture cholesterol. Sections were placed on glass slides and coated with autoradiography emulsion (Sakura: NR-M2). After three weeks exposure, the slides were developed with Konidol X (Sakura) at 20° C for five minutes and stained with Mayer's hematoxylin and eosin. The uptake of cholesterol into the prothoracic glands and the migration of the grains to the hemolymph were examined by counting the grains on two slides per insect and four sections per slide. All grain counts were made under oil immersion at 1250×. Background was less than two grains per 100 μ^2 in all cases and was not subtracted from grain counts. Grain counts were performed in the cytoplasm of the prothoracic glands and the area of hemolymph per 100 μ^2, respectively.

Ecdysone biosynthesis in the prothoracic glands *in vivo* was examined. ^{14}C-cholesterol dissolved in linolenic acid (4.2 μCi ^{14}C-cholesterol/2.5 μl linolenic acid/prepupa) was injected into the diapausing and post-diapausing prepupae, respectively. In other experiments, prepupae were ligated with thread and divided into two parts, the prior part with and the posterior part without prothoracic glands. In each part, ^{14}C-cholesterol was injected at half the previous dosage. In these experiments, after several hours, the parts were homogenized and extracted four times with hot ethyl alcohol (60–70° C) over 24 hours. The extracts were evaporated and the residues dissolved in 20% ethyl alcohol for column chromatography to analyze ecdysone biosynthesis.

Ecdysone biosynthesis in the prothoracic glands also was examined *in vitro*. Thirty pairs of the prothoracic glands of diapausing and post-diapausing prepupae were cultured respectively in the medium CSM-2F (Mitsuhashi, 1968). A small amount of ^{14}C-cholesterol (0.02 mM) was added at the onset of cultivation. After several days, the medium was extracted with hot alcohol, and the residue was dissolved in 20% ethyl alcohol for column chromatography to analyze ecdysone biosynthesis. In some parts, ecdysone biosynthesis was also studied in the prothoracic glands themselves after removing the culture medium by the same methods.

To detect ecdysones, liquid chromatographs were used according to Hori (1969), employing Amberlite XAD-2 (40–50 mesh) and sigmoid gradient elution with 20–90% ethyl alcohol in water at 20° C. As the amount of ecdysones involved in *Monema flavescens* was minute, the fractions of ecdysones were determined at first by the application of pure ecdysones, α-ecdysone and β-ecdysone. The fractions of α- and β-ecdysones were determined by a photometer at 254 mμ after chromatographing with the application of pure α- and β-ecdysones, respectively, before experiments. The fractions of α- and β-ecdysones corresponded to fraction numbers 41 and 51, respectively.

After application of the sample, each fraction was prepared for a scintillation counter. Scintillation fluid consisted of naphthalene 180 g, POPOP 0.5 g, PPO 9 g, ethylene glycol monoethyl ether 500 cc and dioxane 3000 cc. Radioactivity of each fraction was counted with a liquid scintillation counter (Packard).

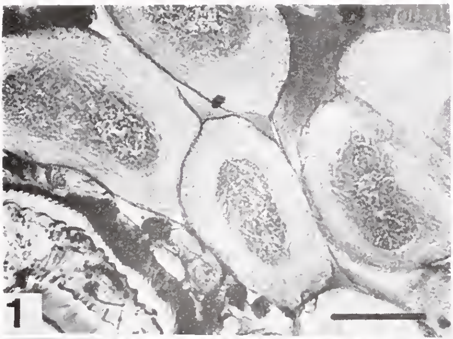
Although it was ascertained photometrically that α-and β-ecdysones corresponded to fractions 41 and 51, respectively, bioassay of these fractions was performed further. The samples were obtained by chromatography from the medium-cultured prothoracic glands of post-diapausing prepupae for five days with addition of nonlabelled cholesterol at the onset of cultivation. The solution of fractions 41 and 51 was concentrated by the evaporator and injected into the diapausing prepupae. After seven days, the state of the insects was observed and compared to controls.

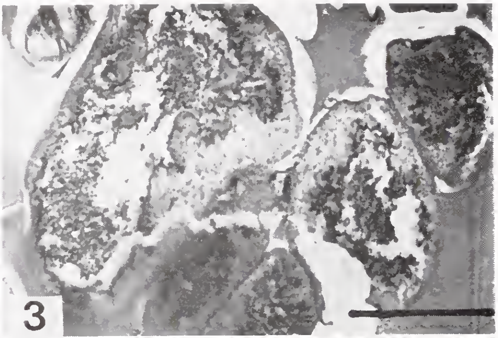
Results

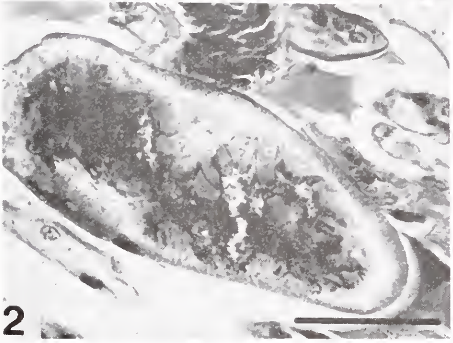
Histological observations of the prothoracic glands with the progress of diapause-break

To clarify the secretion of ecdysone in the stage of diapause-break, the histological changes found in the prothoracic glands with the progress of diapause-break were examined.

Throughout the diapause period, the appearance of the prothoracic gland was almost unchanged. The nucleus was usually regularly spherical or in the shape of a slightly flattened ellipsoid. It appeared to be enclosed in a closely applied membrane, whereas intra-or inter-cellular material was not apparent. The gland cells were spheroid, possessed relatively regular nuclei and inconspicuously striated borders (Fig. 1). The fine structure of the gland cells in the diapause stage is shown in Figure 6. The development of granular-ER was not found. However, many free ribosomes were seen. Lipid droplets and glycogen particles were also seen. In many parts of the gland cells, the presence of undeveloped agranular-ER was clearly observed. This seemed to indicate that the glands have lower activity in this stage and that the synthesis of the secretory substances was suppressed. With the progress of diapause break, striking changes appeared. Initial secretory activity was characterized by the increased nuclear and nucleolar volume that preceded the appearance of the nuclear irregularity. The most striking cytological feature of







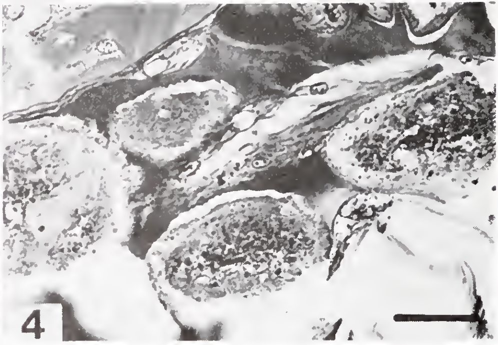


FIGURE 1. Prothoracic glands in the diapausing stage; scale 25 μ.

FIGURE 2. Prothoracic gland at the beginning of nuclear folding; scale 25 μ.

FIGURE 3. Nuclear folding found in the prothoracic glands; scale 25 μ.

FIGURE 4. Prothoracic glands which began to synthesize the secretory substances in the cytoplasm; scale 25 μ.

these glands was the nuclear folding. Figure 2 and Figure 3 show the process of nuclear folding with the progress of diapause-break. Under electron microscopy, the nucleus was enveloped by a double-layered nuclear membrane and contained a few nucleolei, while in the diapause stage, the nucleus was rounded and the folding

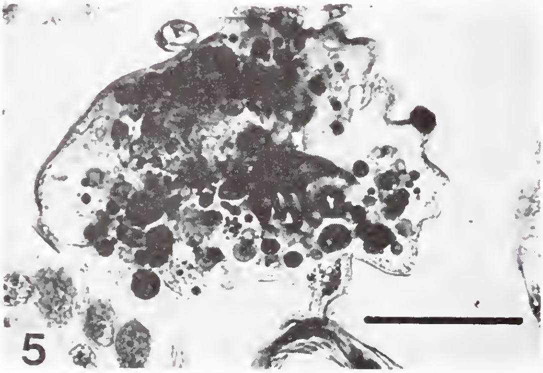


FIGURE 5. Prothoracic gland containing many secretory substances in the cytoplasm; scale 25 μ.

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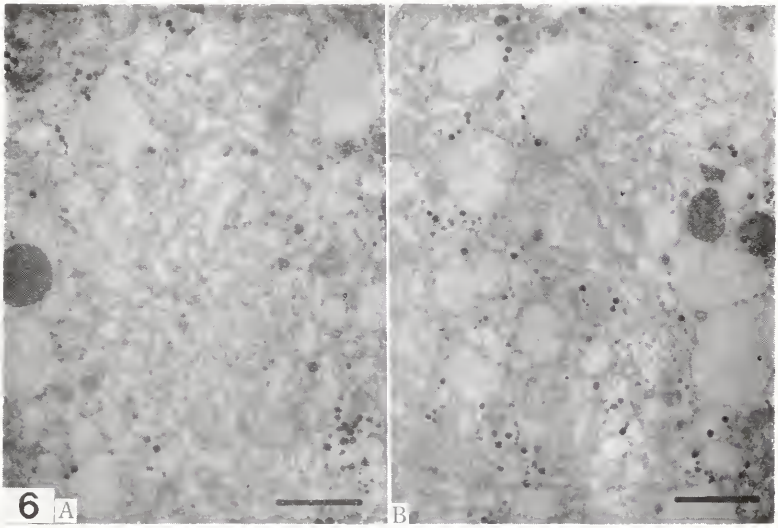


FIGURE 6. (A and B) General views of cytoplasm of the prothoracic gland in the diapausing stage. Undeveloped agranular-ER, lipid droplets, glycogen particles and free ribosomes etc. are seen; scale 1 μ.

did not appear (Fig. 7A). With the progress of the cyclic activity in the gland, the nucleus contained numerous chromatin blocks and few nucleolei and became irregular (Fig. 7B). Then the nucleus began to branch out into the cytoplasm (Fig. 7C). The nuclear folding seems to have an important role in the interaction between the nucleus and the cytoplasm. In this stage, the information necessary for the synthesis of the secretory substances must be transferred from the nucleus to the cytoplasm. After passing this stage, secretory substances began to appear in the cytoplasm of the prothoracic glands (Fig. 4). The secretory substances gradually became large in size and finally the cytoplasm was filled with the secretory substances (Fig. 5). Although the releasing mechanisms were unclear, they were released from the cell to the hemolymph. Agranular-ER, which is believed to synthesize the steroid hormone, began to develop in the peripheral region with the progress of nuclear folding. They were arranged to encircle a relatively narrow intracellular space and formed randomly interconnecting tubes in the peripheral region (Fig. 8). With the progress of the development of agranular-ER, the contact figures between agranular-ER and mitochondria were observed in some places (Fig. 9). This seemed to indicate the transfer of some substances from the agranular-ER to the mitochondria. After that, the deformation of mitochondria was seen in some parts of the cytoplasm. The deformation coincided well with the schematic drawings provided by Wilde (1969). At first, mitochondria were swollen with less dense interiors and their cristae became unclear. Then these organelles were more swollen and some granules formed in them. Thereafter, the number of normal mitochondria decreased gradually and deformed mitochondria made up the greater part of the total mitochondria. This is shown in Figure 10. In the secreting stage, there appeared many secretory granules in the cytoplasm. They may have originated from the mitochondria whose surface membrane had disappeared (Fig. 11). They

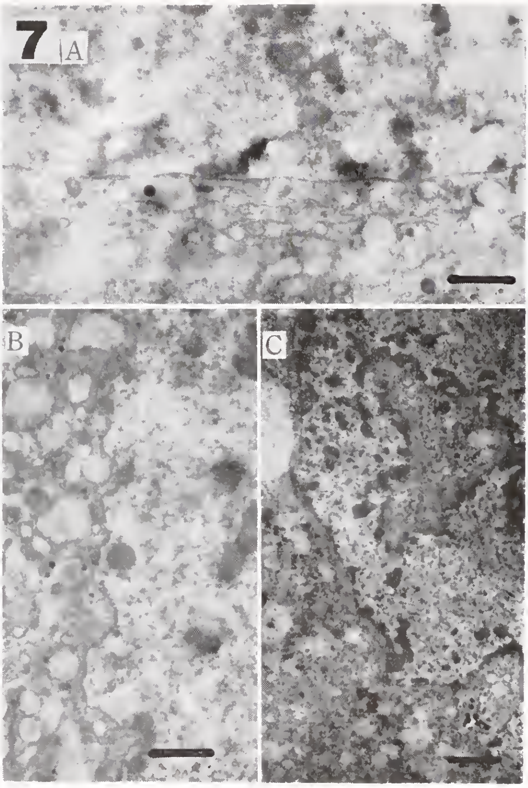


FIGURE 7. A, Appearance of nuclear folding found in the prothoracic glands. The nucleus is rounded and the folding does not appear in the diapausing stage; B, nuclear surface becomes irregular with the progress of activation; C, nucleus then begins to branch out into the cytoplasm; scale 1 μ.

appeared lamellar, granular or nonstructured. Under low magnification, one of them had the appearance of an array of slender filaments more or less parallel to each other. Higher magnification demonstrated that they were actually bundles of minute straight tubules. Nonstructured granules, however, were also present and they occupied the greater part of the secretory substances. From the present figures, the precise relationship between the secretory granules and ecdysone could not be deduced.

Studies were then made of the cell membrane regarding the release of these substances. The peripheral cell membrane of the prothoracic glands showed a form of microvilli (Fig. 12A) and through them the secretory substances seemed to be released. In the releasing stage, some substances always appeared in the peripheral region of the cytoplasm and further in the microvilli (Fig. 12B). The release of these substances was not observed in an intact form. Therefore, some substances found near the microvilli seemed to be the ones synthesized in the mitochondria. It is likely that the intact secretory substances collapsed and were released into the hemolymph through the microvilli in the releasing stage.

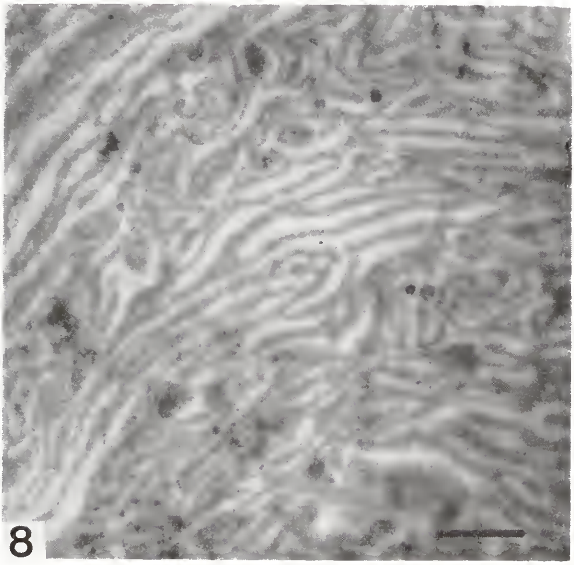


FIGURE 8. Central part of the well developed agranular-ER usually found in the peripheral region in the cytoplasm; scale 1 μ.

The incorporation of tritiated uridine to the prothoracic glands was then examined to clarify the relationship between glandular activity and uridine incorporation. The synthesis of RNA in the prothoracic glands of diapausing prepupae was

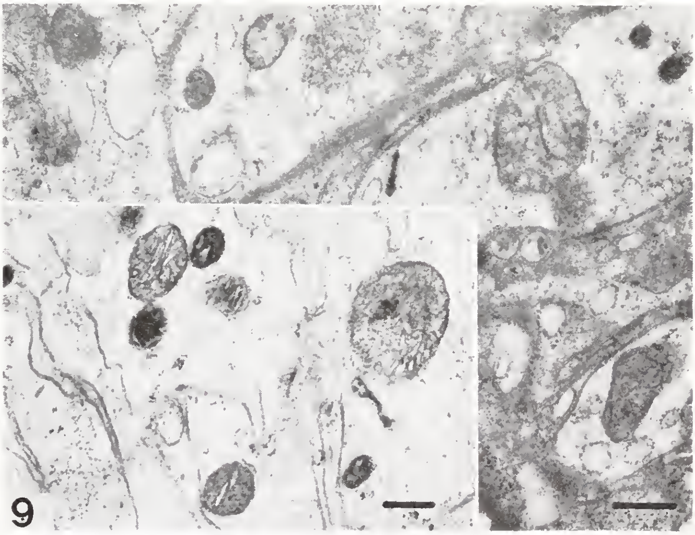


FIGURE 9. Contact figures found between agranular-ER and mitochondria. These are always found in the edge of agranular-ER, away from the central area. Tubules are distinct, different from those of the swollen agranular-ER found in the central part of the mass of agranular-ER; scale 1 μ.

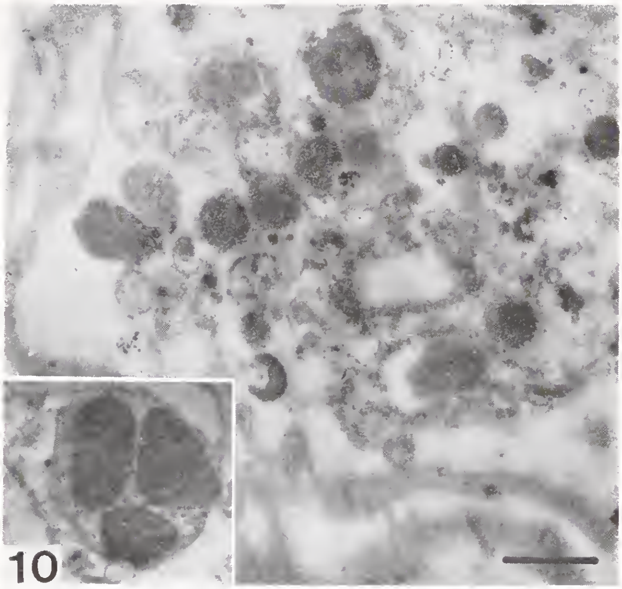


FIGURE 10. Secretory substance synthesis found in the deformed mitochondria; scale 1 μ. Most of the mitochondria found in this stage appeared thus, and resembled the mitochondrial deformation reported by Wilde (1969) for the Colorado beetle.

not found until some factors such as brain hormone activated the glands to secrete ecdysone. When the prothoracic glands were switched from quiescence to secretory activity, there was an increase in the rate of RNA synthesis. The initial low rate

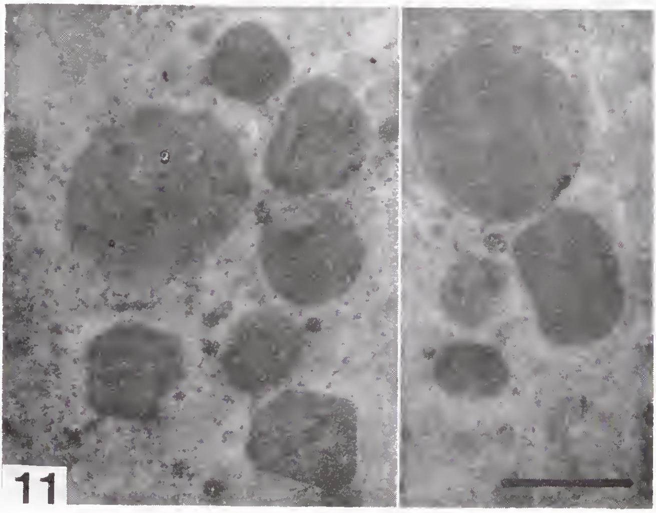


FIGURE 11. Secretory substances found in the cytoplasm. They appear in the cytoplasm after breaking the mitochondrial sheath; scale 1 μ.

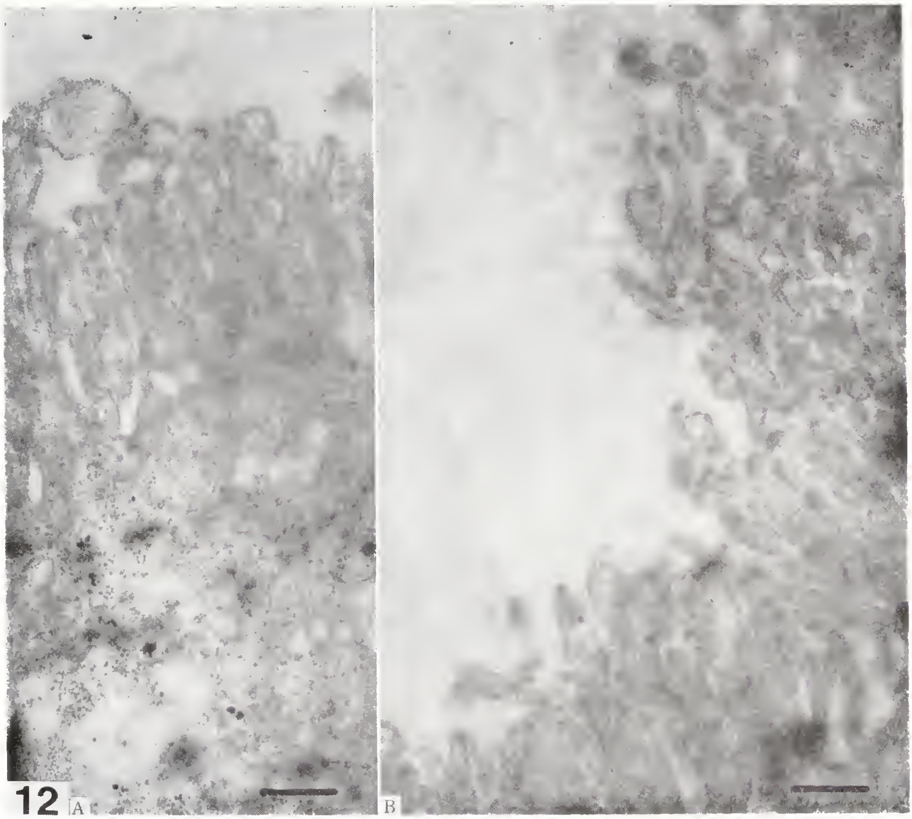
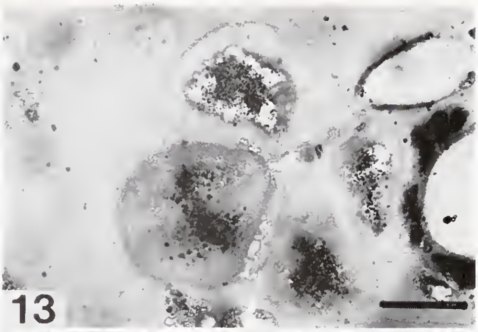


FIGURE 12. A, Microvilli found in the peripheral membrane of the prothoracic glands; B, release of the secretory substances from the microvilli by pinching off tips of microvilli. Dark material to the right of B is made up of condensed secretory substances; scale 0.5 μ.

of RNA synthesis occurred at the time when the prothoracic gland-cell nuclei were regular in shape. By the time the maximum rate of RNA synthesis was reached, nuclear folding and vacuolization were evident. The incorporation was found first in the nucleus (Fig. 13) and then in the cytoplasm (Fig. 14). The correlation between RNA synthesis and the cytological changes during secretion described above was most striking. The increase of uridine incorporation was closely correlated with the appearance of secretory activity in the gland cells. It seems apparent that a close interaction between the nucleus and the cytoplasm is necessary for the synthesis of the secretory substances.

Ecdysone biosynthesis in the prothoracic glands

Detection of cholesterol in the prothoracic glands. Cholesterol has been demonstrated to be the precursor of ecdysone in *Calliphora erythrocephara* (Karlson and Hoffmeister, 1963; Willig *et al.*, 1971), *Calliphora stygia* (Galbraith *et al.*, 1970), *Bombyx mori* (Moriyama *et al.*, 1970; Nakanishi *et al.*, 1972), *Mamestra brassicae*



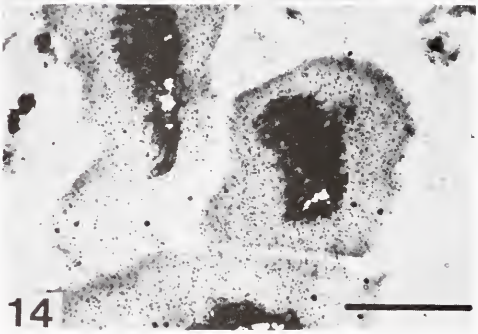


Figure 13. Incorporation of ^{3}H-uridine into the nucleus of the prothoracic glands; scale 25 μ.

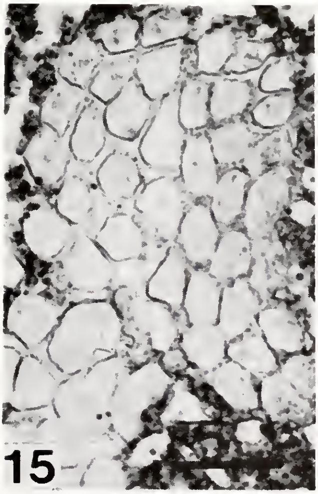
Figure 14. Migration of labelled substances from the nucleus to the cytoplasm; scale 25 μ.

(Gersch and Stürzebecher, 1971), and *Musca domestica* (Studinger and Willig, 1975). Whether the prothoracic glands contained cholesterol was first examined. In *Monema flavescens,* the prothoracic glands showed a positive reaction in the part of their peripheral region (Figs. 16 and 17). As mentioned above, this area coincided with part of the well-developed agranular-ER, which had been shown to contain some enzymes necessary for steroid hormone synthesis from cholesterol (Shikita and Tamaoki, 1964; Clayton, 1969). Positive results were also found in the prothoracic glands of both diapausing and post-diapausing prepupae. It was more prominent in the post-diapausing stage (Fig. 17) than that of the diapausing stage (Fig. 16). The reaction was not uniform in the cytoplasm but always localized in the peripheral region of the gland cells. No detectable cholesterol was found in the muscle tissues used as controls (Fig. 15). The existence of cholesterol is highly significant for ecdysone biosynthesis.

Incorporation of ^{14}C-cholesterol into the prothoracic glands. Since insects are incapable of synthesizing cholesterol, cholesterol found in the prothoracic glands originates in food. The incorporation of cholesterol from the hemolymph to the prothoracic glands was examined by the autoradiography of ^{14}C-cholesterol. In the prothoracic glands of diapausing prepupae, the incorporation was not found, while it was clearly detected in post-diapausing prepupae (Fig. 18). These results were compared further with that of the secretory substance synthesis with the progress of diapause break. With the progress of secretory substance synthesis, the incorporated grains which spread in the cytoplasm gathered each other and formed large grains which corresponded to the secretory substance (Fig. 19) and were released to the hemolymph (Fig. 20). The grains which appeared in the cytoplasm migrated to the hemolymph. They were not released from the cytoplasm in intact clusters of grains, but in small grains (Fig. 20). This agreed with the results of electron microscopy. The time course obtained by counting the grains also showed the results of migration clearly. The amount of grains in the hemolymph increased gradually with the decrease of the grains in the cytoplasm. It is unclear whether the large grains made up of small grains corresponded to the cholesterol molecules or to ecdysone. These results show that cholesterol is incorporated into the prothoracic glands from the hemolymph, and then is secreted into the hemolymph as small grains in response to need for ecdysone.

Ecdysone biosynthesis in the prothoracic glands in vivo. Ecdysone biosynthesis was examined at first in the intact body of the diapausing and post-diapausing prepupae after being injected with ^{14}C-cholesterol. In the diapausing prepupae, ecdysone biosynthesis was not found (Fig. 21, open circle). On the contrary, in the post-diapausing prepupae, high radioactivity was found in the fractions 41 and 51 which were demonstrated to be β-ecdysone and α-ecdysone, respectively (Fig. 21, closed circle). In *Monema flavescens,* the existence of both α- and β-ecdysones were clearly demonstrated *in vivo.*

To examine the synthetic site of ecdysones, ligature treatments were then carried out in the post-diapausing prepupae (Fig. 22). Diapausing prepupae were also used as controls. In the anterior part with the prothoracic glands, α- and β-ecdysones were clearly biosynthesized (Fig. 22, open circle). The amount of β-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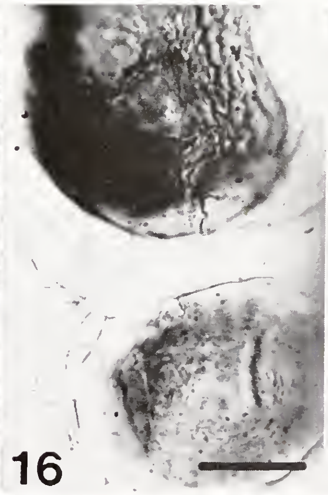
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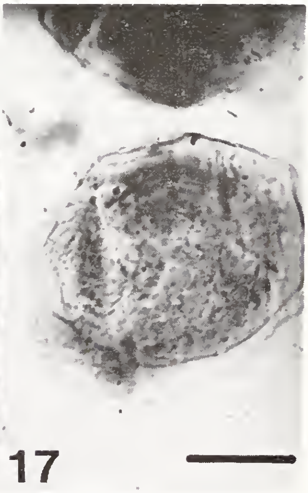
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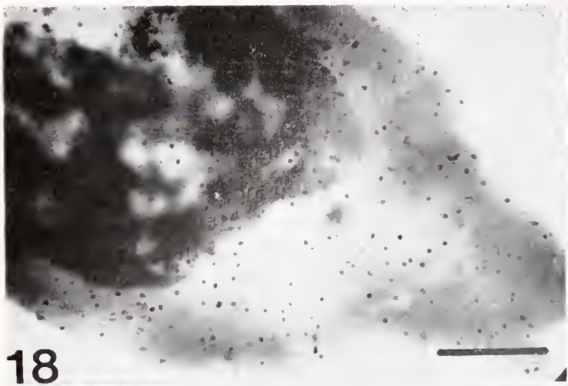
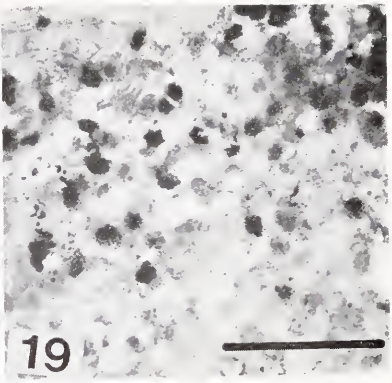
FIGURE 15. Histochemical test for cholesterol in muscle tissue (control). No positive reaction occurs in the whole area of the tissue; scale 25 μ.

FIGURE 16. Histochemical test for cholesterol in the prothoracic glands of diapausing stage. Positive reactions are found in the dark-stained peripheral region of the glands; scale 25 μ.

FIGURE 17. Histochemical test for cholesterol in the prothoracic glands of the post-diapausing stage. Positive reactions are again found in the peripheral region of the glands; scale 25 μ.





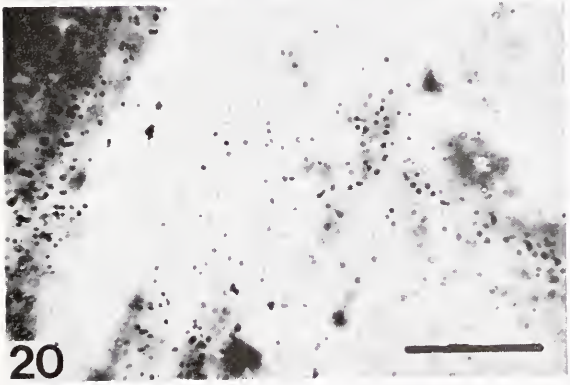


FIGURE 18. Incorporation of ^{14}C-cholesterol into the cytoplasm of the prothoracic glands. Grains are spread throughout the cytoplasm at the beginning of incorporation; scale 10 μ.

FIGURE 19. Clusters of labelled substances appear in the peripheral region of the cytoplasm, and represent the secretory granules; scale 25 μ.

FIGURE 20. Migration of labelled substances from the prothoracic glands (left) to the hemolymph; scale 10 μ.

ecdysone was more than that of α-ecdysone. The radioactivity of ecdysones was not detected in either the anterior part with the prothoracic glands or the posterior part without the prothoracic glands in the diapausing prepupae. Similar results were found in the isolated abdomens of *Bombyx mori* (Moriyama *et al.,* 1970; Nakanishi *et al.,* 1972), *Mamestra brassicae* (Gersch and Stürzebecher, 1971), and *Musca domestica* (Studinger and Willig, 1975). These experiments showed that ecdysone biosynthesis occurred not only in the prothoracic glands but also in the peripheral tissues without prothoracic glands. However, most ecdysones were biosynthesized in the prothoracic glands.

Ecdysone biosynthesis in the prothoracic glands in vitro. To examine the results in the former section, more direct experiments were performed *in vitro.* At first, the prothoracic glands of the post-diapausing stage were cultured in the synthetic medium CSM-2F for five days with the addition of labelled cholesterol at the onset of cultivation. The same treatment was also performed in the prothoracic glands of the diapausing stage as a control. After cultivation, ecdysone biosynthesis

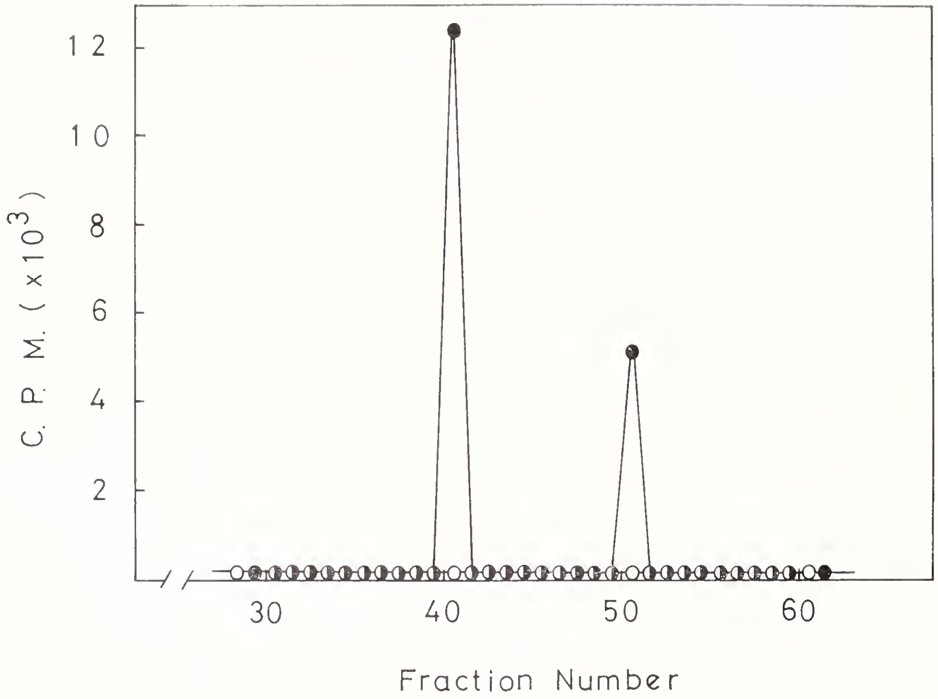


FIGURE 21. Ecdysone biosynthesis in the intact diapausing and post diapausing prepupae. Open circles indicate diapausing prepupae; closed circles, post diapausing prepupae. Fractions 41 and 51 fs correspond to β- and α-ecdysones, respectively. In the post-diapausing prepupae α- and β-ecdysones were clearly biosynthesized *in vivo*.

was examined. In the culture medium of the prothoracic glands of the diapausing stage, ecdysones were not biosynthesized completely. This was not changed by extending the term of cultivation to two weeks. On the other hand, α- and β-ecdysones were clearly biosynthesized in the culture medium of the prothoracic glands of the post-diapausing stage after the same number of days of cultivation (Fig. 23). The results provide the most direct evidence that the prothoracic glands produce and secrete ecdysones. The amount of α-ecdysone was more than that of β-ecdysone, differing from the results *in vivo*. However, in other insects, for example *Bombyx mori* (Chino *et al.*, 1974), *Manduca sexta* (King *et al.*, 1974), and *Leucophaea maderae* (Borst and Engelmann, 1974; King and Marks, 1974), β-ecdysone was not found in the culture medium. The reasons for this are not clear.

Ecdysone biosynthesis and storage was examined further in the prothoracic glands themselves. The prothoracic glands were cultured for five days with the addition of ^{14}C-cholesterol at the onset of cultivation. After that, ecdysone biosynthesis was examined. Before preparation, the prothoracic glands were washed several times in the culture medium CSM-2F. In both the prothoracic glands of diapausing and post-diapausing prepupae, ecdysones were not accumulated in the prothoracic glands themselves. Therefore, it seems likely that the incorporation

TABLE I

Bioassay of the active fractions on the diapausing prepupae of Monema flavescens *seven days after onset of treatment.*

Prothoracic glands	Active fractions	Post-diapausing prepupae (unaffected)	Prepupal-pupal intermediates	Pupae obtained
Post-diapausing stage	41 (β-ecdysone)	0	7	8
	51 (α-ecdysone)	0	13	2
Diapausing stage	41 (β-ecdysone)	6	0	0
	51 (α-ecdysone)	6	0	0
Controls	ethanol	10	0	0

of cholesterol is elaborated to α-ecdysone just before secretion. As α-ecdysone is known to convert into β-ecdysone, β-ecdysone found in the medium seems to originate from α-ecdysone. In the crab, *Hemigrapsus nudus,* however, "labelled β-

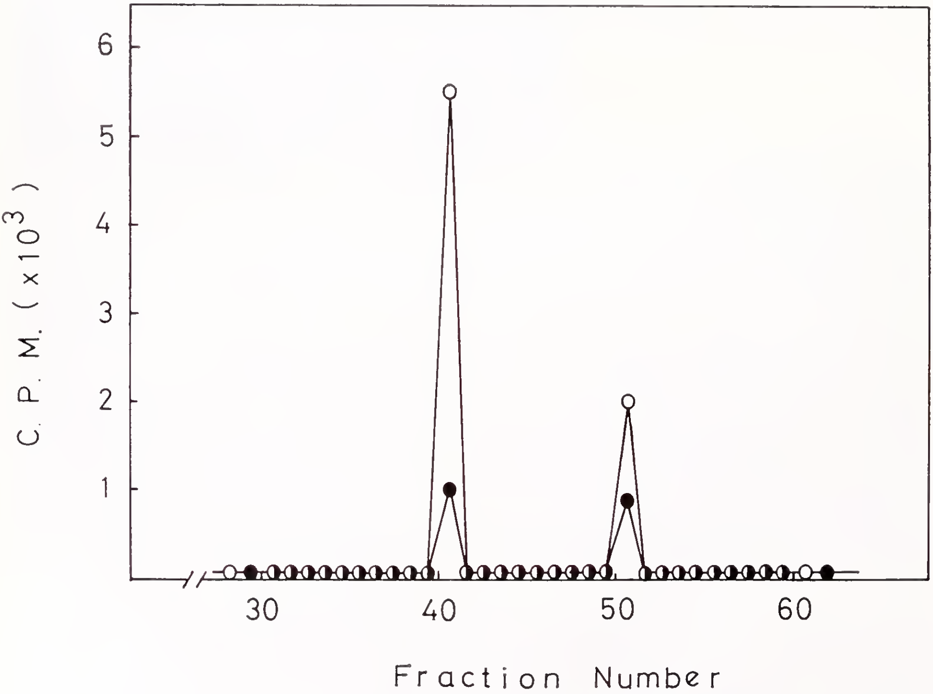


FIGURE 22. Ecdysone biosynthesis in the ligatured post diapausing prepupae. Open circles indicate prior part with prothoracic glands; closed circles, posterior part without prothoracic glands. Fractions 41 and 51 correspond to β- and α-ecdysones, respectively. Even in the posterior part without prothoracic glands, α- and β-ecdysones were biosynthesized *in vivo.*

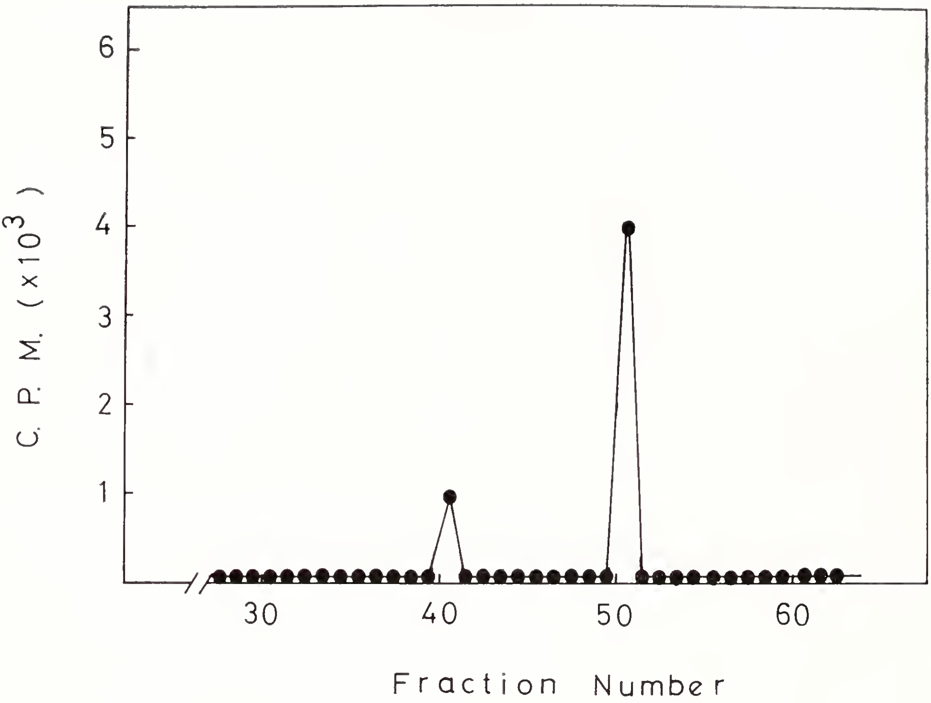


FIGURE 23. Ability of ecdysone biosynthesis in the prothoracic glands of post-diapausing prepupae *in vitro*. Fractions 41 and 51 correspond to β- and α-ecdysones, respectively. Closed circles indicate ecdysone biosynthesis *in vitro*. α- and β-ecdysones were clearly biosynthesized *in vitro*.

ecdysone" was found in the Y-organ itself (Spaziani and Kater, 1973). As described above, α-ecdysone is known to convert into β-ecdysone in *Leucophaea maderae* (King and Siddall, 1969) and in *Bombyx mori* (Moriyama *et al.*, 1970; Nakanishi *et al.*, 1972). In the present experiments, both α- and β-ecdysones were found. However, the amount of β-ecdysone was greater *in vivo* and less *in vitro* than that of α-ecdysone. This seems to result from their conversion.

Bioassay of active fractions. The bioassay of the active fractions obtained by using nonlabelled cholesterol was performed in the diapausing prepupae of *Monema flavescens*. The state of prepupae was examined seven days after injection of each active fraction. The results are shown in Table I. By injecting the active fractions, diapausing prepupae began to change, as the *corpora allata* are active during the diapause period (Takeda, 1970). However, some of them became pupae according to the degree of the activity of the *corpora allata*. Further, it was shown that the activity of fraction 41 was always higher than that of fraction 51. Fractions 41 and 51 correspond to β-ecdysone and α-ecdysone, respectively. This is understood from the fact that β-ecdysone has more active hormonal actions than α-ecdysone. On the contrary, fractions 41 and 51 obtained from the prothoracic glands of diapausing prepupae did not show any biological activities. In the control group using ethanol, biological activities were not found.

Discussion

The fine structure of prothoracic glands containing the developed agranular-ER, mitochondria and lipid droplets is different from that of the glands actively synthesizing the hormone. Well-developed agranular-ER, which may participate in the synthesis of the secretory substances, was found in the prothoracic glands of *Monema flavescens.* King, Aggarial and Bodenstein (1966) have also noticed a considerable increase in smooth-walled vesicles and agranular-ER in the ring gland of *Drosophila melanogaster.* In other insects, agranular-ER was also apparently often found (Locke, 1970). However, they are not always active with relation to ecdysone secretion (Herman, 1967). In rats, the adrenal cortex (Savatini, Robertis and Pleichmar, 1962) and interstitial cells (Christensen and Fawcett, 1961, 1966; Christensen, 1965) that synthesize steroid hormones contain a developed agranular-ER. It is suggested that there is a mutual relationship between the amount of agranular-ER and the amount of steroid, and, further, well-developed agranular-ER is the synthetic site of steroid hormone. In the lutein cells of the rat, agranular-ER is decreased by hypophysectomy and is increased by the supply of the tropic hormone (Enders and Lyons, 1964). Agranular-ER found in the prothoracic glands seemed to be the reaction site for side-chain alteration and hydroxylation of the cholesterol molecules in the course of the elaboration of ecdysone.

Agranular-ER, closely associated with mitochondria, is usually found in the prothoracic glands of *Monema flavescens* before synthesis of the secretory substances. The close relationship between the tubules of agranular-ER and the mitochondria seems to be related to the transference of cholesterol and other substances necessary for steroidgenesis. In some lepidopteran insects such as *Antheraea perni* and *Bombyx mori,* Beaulaton (1964, 1968) also observed the close relationship between the tubules of agranular-ER and macromitochondria. Copeland and Dalton (1959) also observed an association between agranular-ER and mitochondria in the cells of the pseudobranch gland of a teleost fish.

All the mitochondria in the prothoracic glands were spherical and their cristae were in the form of ducts. After contact with agranular-ER, some inclusions occasionally appeared in the mitochondria in the course of their transformation. Wilde (1969) presented schematic drawings of the mitochondrial transformation in the adult Colorado beetle, *Leptinotarsa decemlineata.* This resembled the mitochondria in the prothoracic glands of *Monema flavescens.* Such inclusions have been reported in mammalian steroid-secreting cells such as the testicular interstitial cells (Christensen and Fawcett, 1961, 1966), lutein cells (Enders, 1962; Enders and Lyons, 1964), and adrenocortex (Savatini *et al.,* 1962; Sheridian and Berst, 1967). Electron-dense globular granules and irregular droplets which appeared in the mitochondria might also be concerned with the synthesis of steroid hormone. The sequential transformation of the mitochondria in the prothoracic glands of some lepidopteran insects was investigated by Beaulaton (1968). He showed that essential modification which takes place during each intermolt effects the chondriosome of secretory cells, and he regarded the mitochondria as the initiating site of ecdysone synthesis. Waku and Sumimoto (1969) also regarded the mitochondria in alluring glands of *Bombyx mori* as the starting organelles of the production of secretory substances. Agranular-ER and mitochondria are here considered to be the organelles related to the synthesis of ecdysone.

Lipoidal substances were also found in the prothoracic glands as found in other steroid-secreting cells. The lipid droplets found in the steroid-secreting cells were considered to be the lipid soluble hormones. However, lipid droplets were not synthesized in the testicular interstitial cells of opossum (Christensen, 1965). The lipid droplets in the prothoracic glands may be the stored form of cholesterol which is utilized for the synthesis of ecdysone.

One of the most striking characteristics of the prothoracic glands of *Monema flavescens* is the extreme irregularity of nuclear surface during secretory activity. The nuclear folding may be interpreted as the induction of the transport of the nuclear materials into the cytoplasm. In the prothoracic glands, the irregular outline of the nucleus may be one of the essential characteristics for the synthesis of these secretory substances. It is assumed that the irregular folding of the nuclear surface is necessary for the nucleo-cytoplasmic interaction, by increasing surface area. In the prothoracic glands of *Hyalophora cecropia,* an extremely irregular nucleus was found in the diapausing period (Herman, 1967). At the same time as the nuclear folding, tritium-labelled uridine moves out from the nucleus to the cytoplasm. The synthesis of RNA did not increase in the prothoracic glands of diapausing stage until the brain hormone activated the prothoracic glands to secrete ecdysone. When the glands were switched from the latent state to the active state, this led to an increase in the rate of RNA synthesis, suggesting that the prothoracic glands require RNA synthesis before the synthesis of ecdysone. This nascent RNA seems likely to be utilized to produce enzymes for the synthesis and the secretion of ecdysone. The correlation between the onset of RNA synthesis and the cytological changes was a striking feature. Similar results were also obtained in the prothoracic glands of *Antheraea polyphemus* (Oberlander, Berry, Krishnakumaran and Schneiderman, 1965).

Regarding the release of the secretory substances from the prothoracic glands, there are no earlier investigations. In *Hyalophora cecropia,* a thick peripheral membrane surrounds each cell and sends numerous invaginations into the gland cell cytoplasm (Herman, 1967). On the contrary, in *Monema flavescens,* the cell membrane showed a form of microvilli. In the releasing stage, the large secretory granules degenerated into many small particles. In *Papilio xuthus,* the secretory granules in the prothoracic glands are known to become a colloid for release through the cell membranes (Yashika and Yoshizaki, 1967). In *Monema flavescens,* these collapsed secretory granules containing ecdysone were released from expanded tips of the microvilli. This extrusion corresponds to Type III as proposed by Kurozumi and such phenomenon are usually found in the choroid plexus, the carpal organ of the pig and the interhepatic bile duct (Kurozumi, 1961).

The present results provide direct evidence of ecdysone biosynthesis in the prothoracic glands of *Monema flavescens.* Since the preliminary report that the prothoracic glands secrete ecdysone *in vitro* have appeared (Takeda, 1972a), many investigators have tried similar experiments independently. The metabolism of cholesterol in the ecdysal glands was also studied in the Y-organ of the crab, *Hemigrapsus nudus* (Spaziani and Kater, 1973). They investigated the uptake and turnover of ^{14}C-cholesterol in the Y-organ and obtained labelled "ecdysone-like metabolites," β-ecdysone, at 24 hours after injection of labelled cholesterol. Galbraith *et al.* (1970) showed that 7-dehydrocholesterol, which is found in relatively high concentration in insect prothoracic glands (Robbins, Thompson, Kaplanis and Shortino, 1964), is not incorporated into β-ecdysone in *Calliphora stygia* with significantly more efficiency than is cholesterol itself. In *Musca domestica,* Studinger and Willig (1975) reported that the radioactivity of labelled cholesterol is concentrated in some parts of the fat body and especially in the oenocytes. They postulated that the oenocytes play a central role in the metabolism and storage of sterols. King *et al.* (1974) cultured the prothoracic glands of *Manduca sexta,* examined the secretory substance and determined it to be α-ecdysone. Chino *et al.* (1974) also identified the secretory substance as α-ecdysone by using the prothoracic glands of *Bombyx mori, in vitro.* Similar results were also obtained in *Leucophaea maderae* (King and Marks, 1974; Borst and Engelmann, 1974), and *Tenebrio molitor* (Romer *et al.,* 1974).

In the ligature experiments of post-diapausing prepupae of *Monema flavescens,* the posterior part without the prothoracic glands also showed the ability of ecdysone biosynthesis as well as the anterior part with the prothoracic glands. When the prepupae were ligated, ecdysone had already been secreted. Therefore, ecdysone biosynthesis found in the posterior part seemed to be the result of some substances contained in the peripheral tissues or secreted from the prothoracic glands with ecdysone. In a preliminary report (Takeda, 1972a) the possibility was suggested that the active prothoracic glands secrete some enzymes or related substances which catalyze and elaborate one or more steps necessary for ecdysone biosynthesis. Nakanishi *et al.* (1972) have evidence which suggests that α-ecdysone can be synthesized from cholesterol and is converted into β-ecdysone in isolated abdomens of *Bombyx mori* without the prothoracic glands. Gersch and Stürzebecher (1971) demonstrated that ecdysone biosynthesis occurs in the isolated abdomens of *Mamestra brassicae.* Further, the abdomens of *Musca domestica* are also shown to synthesize α- and β-ecdysone (Studinger and Willig, 1975). Ecdysone biosynthesis outside the prothoracic glands can result from some enzymes or related substances secreted from the prothoracic glands with ecdysone in their former active stage.

The present results are explained as follows with special reference to ecdysone synthesis. Initial secretory activity of the prothoracic glands is characterized by increased cell and nuclear volume. The information necessary for the synthesis of secretory substances is transferred from the nucleus to the cytoplasm by the nuclear folding. Cholesterol molecules are then incorporated into the cytoplasm, transferred from agranular-ER to the mitochondria, and incorporated into the secretory substances of the mitochondria. In the course of this transference, cholesterol molecules are elaborated to the precursor of ecdysone. Some granules which are contained in the agranular-ER and the mitochondria and are able to synthesize ecdysone from cholesterol also consist of secretory substances. After breaking the mitochondrial sheath, the secretory substances collapse in the cytoplasm and are released through microvilli to the hemolymph. Just before the secretion, the precursor of ecdysone is elaborated to α-ecdysone and is secreted into the hemolymph where α-ecdysone is converted to β-form.

The author wishes to express his thanks to the late Prof. M. Fukaya for his continuous guidance and encouragement throughout the course of these experiments.

SUMMARY

The problem of hormonal secretion with special reference to the synthesis of ecdysone in the prothoracic glands of *Monema flavescens* (Lepidoptera) was investigated.

Initial secretory activity of the prothoracic glands is characterized by increased cell and nuclear volume, and extensive nuclear folding. Cholesterol molecules are incorporated into the cytoplasm. They are transferred from the agranular-ER to the mitochondria and incorporated into the secretory granules of the mitochondria. In the course of transference, cholesterol molecules are elaborated to the precursor of ecdysone. After breaking the mitochondrial sheath, the secretory material breaks down in the cytoplasm and is released through microvilli to the hemolymph. At that time, the precursor of ecdysone is elaborated to α-ecdysone and is then secreted into the hemolymph where α-ecdysone is converted to the β-form.

In the present experiments, the prothoracic glands were demonstrated to synthesize ecdysones from cholesterol and secrete them. These facts were demonstrated both *in vivo*, and *in vitro*, in cultured prothoracic glands.

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HYDRATION IN THE SAND SHRIMP *CRANGON SEPTEMSPINOSA*: RELATION TO DIET

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The sand shrimp *Crangon septemspinosa* Say 1818 is a common inhabitant of estuaries along the northwestern Atlantic from Newfoundland to eastern Florida (Squires, 1965; Williams, 1965; Price, 1962). The biology and ecology of the species is discussed by Embich (1973), Haefner (1969a, 1969b, 1970, 1971, 1972, 1973), Price (1962), Regnault (1970, 1971, 1972), Regnault and Costlow (1970), Sandifer (1975), Wilcox (1972), Wilcox and Jeffries (1973, 1974).

Hydration levels of crustaceans are affected by a variety of environmental and physiological parameters, and the literature detailing these factors is extensive (*e.g.,* Passano, 1960; Roberston, 1960). For example, molting of crustaceans has been scientifically documented for almost fifty years, and its endocrinology and ancillary process of growth is known in detail (Hoar, 1975).

The literature dealing with hydration in *Crangon* spp. is, however, quite limited. To document water content over a molt cycle, a generalized account suggested by Lockwood (1967) must be used. Thus, water content can vary from a high of 86% immediately after ecdysis (Drach Stage A_2) to a low of 59–61% during Drach Stage D. The time spent at high levels of hydration (greater than 80% water) is approximately 10% of a molt cycle. Weber and van Marrewijk (1972) negatively correlated percentage water content of muscle in *Crangon crangon* to increasing environmental salinity and determined that the shrimp possesses a relatively efficient water regulation system. Haefner (1969b) determined the osmoregulatory patterns of *C. septemspinosa* and found that all life stages were hyperosmotic in a dilute medium (15‰): adults were isosmotic and juveniles were hypoosmotic in 45‰; and all life stages were hypoosmotic in 30‰ sea water. Cuzon and Ceccaldi (1973) determined that starvation affects the hydration levels of *C. crangon* and that percentage water of muscle varied from 76.1 ± 1.4% to 77.7 ± 0.5% over the four-week study.

Adequacy of foods and their utilization are revealed by several indicies in *C. septemspinosa.* Growth of the shrimp on a variety of diets over a period of weeks has been discussed by Wilcox and Jeffries (1974). Freshly prepared foods (*e.g.,* the clam *Mercenaria mercenaria,* the brine shrimp *Artemia salina,* hard-boiled egg) promoted better growth than dried foods (*e.g., Mercenaria, Artemia, Crangon,* fish meal, copepods). This difference in growth was attributed to loss of olfactory attractants upon drying. In this study, the effects of nutrition on hydration in *C. septemspinosa* were determined.

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Materials and Methods

In all experiments to relate hydration to nutritional status, shrimp of both sexes and greater than 25 mm in length were seined from Pettaquamscutt River, Narragansett, Rhode Island (41° 26′ 55″ N and 71° 27′ 05″ W; water temperature 20° C; salinity 27–29‰) during the summer and fall of 1970 and 1971. Two laboratory groups were placed in 300-liter tanks with a sand substratum. Sea water (20° C, 30–32‰) from Narragansett Bay was filtered through glass wool to remove particulate material and was circulated through the tank at 1 liter/min. All other experiments had the same water source and flow rate. In one laboratory group of 29 shrimp, fresh tissues of *Mercenaria* were given *ad libitum* for a total of seven days (fresh tissue: *Mercenaria* opened 15 min before feeding and all soft tissue diced). Uneaten portions were removed prior to the daily feeding period. No food was given to the second laboratory group of 50 shrimp. A third group of 80 shrimp collected in the field and analyzed for hydration served as control.

Shrimp were blotted to dryness on paper towels, individually weighed to the nearest 0.1 mg (wet weight), freeze dried, and then reweighed. Hydration was expressed as a percentage of the dry weight to wet weight.

Fisher's Least Significant Difference (LSD) was used for pair-wise comparison of groups with multiple means (Fryer, 1966). If two means differed by the LSD, they were statistically different at $P = 0.05$. Student's t-statistic for unequal variances was used for pair-wise comparison of two means (Fryer, 1966).

Further hydration-feeding experiments were made with 225 shrimp held in a tank (2 × 0.25 × 0.25 m) divided into 12 compartments, each with a sand substratum. This experimental design allowed four replicates of the three feeding regimes. Filtered sea water entered one end and drained at the opposite end. Diets were as follows: 2.2% agar in sea water (Bactoagar, Difco Laboratories, Detroit, Michigan): homogenized freeze-dried tissues of *Mercenaria* in agar (6 g tissue in 30 ml of 2.2% agar); and no food. Each diet was prepared as a batch and smaller portions were given *ad libitum* on a daily basis for seven days.

In a final set of hydration-feeding experiments, 14–24 shrimp were held in 300-liter tanks with a sand substratum, a screen partition dividing the tanks in half (control group on one side and experiment group on the other), filtered sea water, and daily *ad libitum* feeding. Control diet for each trial was fresh tissues of *Mercenaria*. Each experimental diet was prepared as a batch by mixing 6 g of dried tissue in 30 ml of 2.2% agar; smaller portions of the diet were then fed to the shrimp for fourteen days. The amount of food ingested in each trial was estimated by the amount removed prior to the next feeding period. Foods in the experimental diets were as follows: 1) soft tissues of *Mercenaria* homogenized and freeze-dried; 2) fish meal of hake (*Urophycis* spp.) obtained from Point Judith, Rhode Island; 3) *C. septemspinosa* taken from Pettaquamscutt River, freeze-dried, and ground with a mortar and pestle; 4) blocks of frozen adult *Artemia* obtained from Metaframe Co., San Francisco, California and freeze-dried; 5) calanoid copepods collected over a period of a year in Narragansett Bay and freeze-dried; 6) beef liver purchased at a supermarket, homogenized, and freeze-dried; 7) 2.2% agar made in sea water; 8) marine yeast (*Candida sake*) isolated from macrovegetation in tide pools at Narragansett, Rhode Island and grown in a malt extract broth (25 g/liter sea water), and freeze-dried; 9) Baker's yeast obtained from

TABLE I

Hydration levels of C. septemspinosa *were determined after seven days of feeding. The LSD for each combination of pairs was: $LSD_{12} = 1.03$; $LSD_{13} = 0.86$; $LSD_{23} = 1.11$;* P *= 0.05. The environmental parameters were: water temperature 20° C; laboratory salinity 30–32 ‰; field salinity 27–29 ‰.*

Group	Food	x̄ ± s.d. Per cent water	N
1 Field	Natural diet	70.74 ± 2.66	80
2 Laboratory	Fresh tissues of *Mercenaria*	72.74 ± 2.36	29
3 Laboratory	Starved	74.84 ± 1.91	50

Fleishmann's Yeast, Standard Brands, Inc., New York; 10) bacteria, probably of a *Pseudomonas* group, isolated from Narragansett Bay sea water and grown in 1.0 g yeast extract and 1.0 g trypticase/liter sea water; 11) TetraMin (dried tropical fish food) obtained from TetraKrafteWerke, West Germany; 12) *Spartina alterniflora* detritus and attached microflora, 0.500–0.017 mm particle size, collected at Bissels Cove marsh in North Kingston, Rhode Island.

In an ancillary experiment to determine hydration levels of recently molted shrimp, males and nonovigerous females of greater than 25 mm in length were collected during July 1970 from Pettaquamscutt River. Each shrimp was placed in an individual 10-liter container provided with sand substratum, unfiltered sea water, and fresh food (tissues of *Mercenaria*) given *ad libitum.* Nine shrimp were analyzed for water content within 24 or 48 hrs of molting; 23 individuals that had not molted in the last 96 hrs served as control.

RESULTS

Significant differences in hydration were found among a field group, a laboratory group that was fed, and a laboratory group that was starved (Table I: LSD, $P = 0.05$). The difference between the field group and the laboratory-fed group could be attributed either to environmental differences or to capturing, handling, and keeping. Because both laboratory groups were maintained under identical conditions, the difference in hydration levels between Group 2 and 3 was presumably due to lack of food.

Groups fed fresh tissues of *Mercenaria* as opposed to freeze-dried tissues of *Mercenaria* in agar had no significant differences (Table II: Trials 1–3, LSD, $P = 0.05$). Due to varying natural salinity during these trials, results should not be compared between one trial and another. In Trial 4 (Table II), there was a significant difference between the fed groups (freeze-dried *Mercenaria* in agar as opposed to agar; LSD, $P = 0.05$), and a difference (LSD, $P = 0.10$) between the group fed tissues of freeze-dried *Mercenaria* in agar and the starved group. However, there was no significant difference in hydration (LSD, $P = 0.05$) between the group fed agar and the starved group. Approximately 75% of the agar fed to the shrimp in Trial 4 was recovered prior to the next feeding period. Thus, the shrimp ingested only small amounts of this food on a daily basis, and this group, for all practical purposes, was subjected to nearly identical conditions as the starved group. Therefore, elevated levels of hydration appear to be related to low levels of ingestion or complete lack of food (Table II: agar, Trial 4; starved group, Trials 1–4).

In another experiment (Table III), foods for Trials 1–12 were selected which would be similar to those available in nature. Sand shrimp which were fed tissues of marine organisms (*i.e., Mercenaria,* fish meal, *Crangon, Artemia,* copepods) did not hydrate, while shrimp fed foods from terrestrial, plant, or microbial sources (*i.e.,* beef liver, agar, marine yeast, Baker's yeast, bacteria, fish food, and *Spartina* detritus) did hydrate. On a short-term basis, marine foods (*i.e.,* foods of Trials 1–5) were ingested to a greater extent than the foods of Trials 6–12. Again, elevated levels of hydration appear to be related to low levels of ingestion.

Hydration ($\bar{x} \pm$ s.d.) in shrimp that had molted in the last 24 and 48 hrs was 77.71 ± 1.24% (Group 1, n = 6) and 76.85 ± 0.41% (Group 2, n = 3). Shrimp that had not molted in the last 96 hrs had hydration levels of 71.70 ± 2.42% (control; Group 3, n = 23). The LSD for each combination of pairs was $LSD_{12} = 3.30$, $LSD_{13} = 2.14$, $LSD_{23} = 2.86$ ($P = 0.05$). Hydration, therefore, in recently molted shrimp was significantly greater than the control. Even after 48 hrs hydration was elevated but declining from a post-molt high of 77.71%.

DISCUSSION

Hydration of *C. septemspinosa,* as well as any other crustacean, is affected by many physiological processes. High levels of hydration occur after ecdysis and

TABLE II

Hydration levels of C. septemspinosa *were determined after seven days of feeding. The clam and agar diet was freeze-dried tissues of* Mercenaria *in agar and the clam diet was fresh tissues of* Mercenaria. *The LSD was calculated at* P = *0.05. The environmental parameters were: water temperature 20° C; salinity 30–32 ‰.*

Food	N	x̄ ± s.d. Per cent water	LSD
Trial 1			
Clam (control)	24	72.47 ± 1.85	
Clam + agar	24	72.65 ± 1.59	1.01
Starve	24	74.30 ± 1.78	
Trial 2			
Clam (control)	22	70.44 ± 2.07	
Clam + agar	22	70.75 ± 1.93	1.16
Starve	22	73.32 ± 1.72	
Trial 3			
Clam (control)	20	74.58 ± 2.42	
Clam + agar	20	74.85 ± 1.28	1.25
Starve	20	76.33 ± 2.03	
Trial 4			
Clam + agar (control)	19	74.66 ± 2.41	
Agar	19	76.88 ± 2.63	1.82
Starve	19	76.33 ± 3.20	

Table III

Hydration levels of C. septemspinosa *were determined after fourteen days of feeding. The control food was fresh tissues of* Mercenaria. *See the text for the source and the preparation of the foods. The level of significance was*: $P < 0.05$, $P < 0.01$, *and ns* (*not significant*). *The environmental conditions were: water temperature 20° C; salinity 30–32 ‰.*

Trial	Food	x̄ ± s.d. Per cent water	DF	*t*-statistic	Level of significance
1	Control	74.35 ± 2.17	16	1.06	ns
	Freeze-dried *Mercenaria*	73.39 ± 1.66			
2	Control	71.72 ± 2.25	18	0.66	ns
	Fish meal (dried)	72.29 ± 1.58			
3	Control	71.10 ± 2.64	20	0.04	ns
	Freeze-dried *Crangon*	71.05 ± 2.61			
4	Control	72.25 ± 3.10	24	1.42	ns
	Freeze-dried *Artemia*	73.83 ± 2.51			
5	Control	74.61 ± 2.79	14	1.43	ns
	Freeze-dried copepods	76.08 ± 0.87			
6	Control	70.87 ± 3.05	20	2.09	≤0.05
	Freeze-dried beef liver	73.30 ± 2.36			
7	Control	70.44 ± 2.54	22	7.08	≤0.01
	Agar	76.26 ± 1.30			
8	Control	70.83 ± 2.54	22	5.46	≤0.01
	Freeze-dried marine yeast	76.14 ± 2.21			
9	Control	72.59 ± 3.26	16	2.97	≤0.01
	Baker's yeast (dried)	76.35 ± 1.94			
10	Control	70.62 ± 2.56	16	6.76	≤0.01
	Freeze-dried marine bacteria	77.82 ± 1.91			
11	Control	70.72 ± 1.56	16	5.47	≤0.01
	TetraMin (dried fish food)	75.82 ± 2.32			
12	Control	74.11 ± 2.92	18	5.74	≤0.01
	Freeze-dried *Spartina* detritus	79.81 ± 1.20			

decrease 48 hrs following a molt. Lockwood (1967) and Passano (1960) also determined that maximum levels of hydration occurred immediately after ecdysis and were of short duration, whereas low levels of hydration occurred during the remainder of the molt cycle.

Ecdysis and its attendant cycle of hydration is a variable that can never be satisfactorily factored out of an experiment. The problem, however, can be minimized by using a sufficient sample size. Since a crustacean spends approximately 10% of its molt cycle at elevated water levels (Drach Stage A_2; Lockwood, 1967), the chances of a control or experimental shrimp being harvested in Drach Stage A_2 are approximately 1 in 10. The number of shrimp used in each experiment was 14–80. Statistical presentation of hydration-feeding results would, therefore, minimize the confounding effects of ecdysis.

Meixner (1969) observed that a rise in water temperature accelerated growth, as well as the molting rhythm of *C. crangon*. Wilcox and Jeffries (1973) concluded that growth rate is a positive function of water temperature and a negative function of size (*i.e.*, larger shrimp grow slower than smaller shrimp). For instance, Wilcox (1972) reported that at an average water temperature of 13° C the intermolt period of *C. septemspinosa* was as follows: 2 weeks for 15–29 mm length shrimp; 3 weeks for 30–39 mm shrimp; and 4 weeks for 40–49 mm shrimp. The size of shrimp used in the present experiments was greater than 25 mm and the water temperature was 20° C. The test period was short (1 or 2 weeks) compared to an extrapolated 2–3 week intermolt period for 25 mm or larger shrimp at 20° C. Therefore, although size and water temperature affect the molting cycle in *Crangon*, by selecting a short experimental period, the confounding effects of the molt cycle may also be minimized.

Hydration of *C. septemspinosa*, upon transfer from field to laboratory (*i.e.*, a salinity increase; Table I), is not simple osmosis, because internal water content would be expected to decrease on transfer to higher salinity (Hoar, 1975). Moreover, since Weber and van Marrewijk (1972) have determined that *C. crangon* is an efficient regulator in the range of salinity encountered, minor salinity changes (*ca.*, 3‰) would probably not tax water regulation of *Crangon* spp. In this case, perhaps hydration is a response to capturing, handling, and laboratory maintenance.

Hydration could result either from lack of nutrient input, or the inability of the shrimp to digest and metabolize the ingested food. Wilcox and Jeffries (1974) have suggested that the limitation of feeding for *C. septemspinosa* lies not with its ability to digest the food, but with the stimulated response of food location and ingestion. Based on our present results, starvation or low food input is a probable explanation for hydration. Examples of "poorly ingested" foods would be agar (Table II: Trial 4) and foods from terrestrial, plant, or microbial sources (Table III: Trials 6–12). Shrimp presented these foods, on a short-term basis, are presumably on a marginal existence and catabolizing metabolic reserves. Thus these shrimp, for all practical purposes, respond the same as starved shrimp.

Foods from terrestrial, plant, or microbial origins, perhaps, lacked water-soluble components that attract shrimp and stimulate ingestion. Observations by Wilcox (1972) on the feeding behavior of *C. septemspinosa* showed that they located food in a homing zig-zag path (klinotaxis: see Hoar, 1975, p. 500). Similar types of homing behavior are also common for other crustaceans (Pardi and Papi, 1961). Other investigators feel that initiation of feeding chiefly depends on chemical stimuli (Barber, 1961; Nicol, 1967). Lindstedt (1971), for example, found that two chemical activators were necessary for a complete feeding response in the sea anemone. One activator initiated contraction and bending of tentacles, the other controlled ingestion. Gurin and Carr (1971) concluded that specific proteins were necessary to induce feeding in the marine snail *Nassarius obsoletus*. Drying, either by heat or freeze-drying, apparently modifies the food, as suggested by Wilcox and Jeffries (1974). Sublimation or heat, besides removing water, may have removed or altered sensory attractants, which stimulate feeding. Foods of a terrestrial, plant, or microbial origin may have been more effectively stripped of attractants by freeze-drying, if the attractants were present at all.

Regnault, Campillo, and Luquet (1975) reported better survival and weight increases of the shrimp *C. crangon* and *Palaemon serratus* when fed a hydrated artificial diet (pastes) contrasted to pelleted artificial foods. They also felt that the hydration level of the diet may have an influence on the ingestion rates for these shrimp.

Based on the results of the hydration experiments (Tables I–III), tissues of *Mercenaria*, either fresh or freeze-dried, provided nutrients to *C. septemspinosa*. Similarly, Wilcox and Jeffries (1974) concluded that these two foods provided nutrients to the shrimp on a long-term basis. There was a difference, however, in growth performance between the fresh and dried diets, which was attributed to olfactory quality.

If starved or given agar (Tables I–III), the shrimp hydrate in a short period of time. Wilcox and Jeffries (1974) demonstrated that agar provided nutrients to *C. septemspinosa* but at a lower rate when contrasted with foods derived from marine sources. Wilcox (1972) also noted hepatopancreal shrinkage in *C. septemspinosa* during starvation. Cuzon and Ceccaldi (1973) observed that starved *C. crangon* metabolized glucides first, lipid reserves next, and proteins last. Hydration of muscle tissue increased over the experiment. Presumably as food reserves are metabolized and tissues shrink, water replaces tissues to maintain internal turgidity.

Although terrestrial, microbial, and plant foods of Trials 6–12 (Table III) caused hydration of *C. septemspinosa* on a short-term basis (two weeks), Wilcox and Jeffries (1974) showed that these same foods provided nutrients sufficient for growth and survival over eight weeks, but at reduced rates when contrasted to foods derived from marine sources. Also, the maintenance of *C. septemspinosa* feeding on some of these same foods may have been marginal since Wilcox (1972) reported hepatopancreal atrophy. Additionally, starvation of *Crangon* spp. can still result in molting and growth (Lockwood, 1967; Wilcox and Jeffries, 1974). Thus, the difference in response on identical foods may reflect the sensitivity of the hydration/growth indices.

Nutritional experiments using growth and survival data as criteria of performance require long-term studies. Even lengthy studies may be misleading, because some crustaceans can continue to grow under nutritional stress. Perhaps egg-laying capacity would be an equivalent or more sensitive long-term index (Lockwood, 1967). Hydration, however, may be a short-term, sensitive, and expedient indicator of nutritional status in *C. septemspinosa* and other crustaceans.

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SUMMARY

Tissue hydration in the estuarine sand shrimp *Crangon septemspinosa* is correlated with nutritional conditions. Hydration levels of shrimp who are ingesting

food remain normal. In starved shrimp or in individuals who are not ingesting adequate amounts of food, hydration levels are high. Presumably, water replaces metabolized tissues.

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